ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 February 28; 9(1): 1-100



Published by Baishideng Publishing Group Inc

WJMA

World Journal of Meta-Analysis

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World Journal of Meta-Analysis

Bimonthly Volume 9 Number 1 February 28, 2021

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AIMS AND SCOPE

The primary aim of *World Journal of Meta-Analysis* (*WJMA*, *World J Meta-Anal*) is to provide scholars and readers from various fields of clinical medicine with a platform to publish high-quality meta-analysis and systematic review articles and communicate their research findings online.

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INDEXING/ABSTRACTING

The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Saurabh Chandan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2308-3840/editorialboard.htm

PUBLICATION DATE
February 28, 2021

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https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 February 28; 9(1): 1-11

DOI: 10.13105/wjma.v9.i1.1

ISSN 2308-3840 (online)

EVIDENCE REVIEW

Inflammatory pseudotumor-like follicular dendritic cell sarcoma: Literature review of 67 cases

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Supported by National Natural Science Foundation of China, No. 81570509.

Conflict-of-interest statement: The authors declare having no conflict-of-interest.

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Abstract

Inflammatory pseudotumor (IPT)-like follicular dendritic cell (FDC) sarcoma is rare. The 2017 World Health Organization classification of tumors of hematopoietic and lymphoid tissues noted that data on its clinical outcome are limited, but that the tumor appears to be indolent. The aim of this study was to summarize the clinical characteristics, treatment outcomes, and prognostic factors for IPT-like FDC sarcoma. A literature review was conducted on retrospective analyses of clinical data and prognostic information on IPT-like FDC sarcoma reported between 2001 and 2020. A total of 67 cases of IPT-like FDC sarcoma were retrieved from the literature, documenting that it occurs predominantly in middle-aged adults, with a marked female predilection. Six patients had a separate malignancy and five had an autoimmune disease. Typically involving the spleen and/or liver, it may also selectively involve the abdomen, gastrointestinal tract, pancreas, retroperitoneum, and mesentery. Necrosis, hemorrhage, noncaseating epithelioid granulomas, and fibrinoid deposits in blood vessel walls are often present. The neoplastic cells are predominantly positive for follicular dendritic cell markers such as cluster of differentiation 21 (CD21), CD23, CD35 and CNA.42 and are consistently Epstein-Barr virus (EBV)-positive. Mitoses were very rare in most cases. Most patients were treated by surgery alone. Disease status at the time of last follow-up was known for 57 patients with follow-up time ranging from 2 to 144 mo. Local and/or distant recurrence after initial treatment was seen in 15.8% of the patients. The 1- and 5-year progression-free survival for the entire group was 91.5% and 56.1%, respectively. Kaplan-Meier and multivariate analyses showed that age, sex, tumor size, and pathological features were not risk factors for disease progression. IPT-like FDC sarcoma appears to be mildly aggressive and requires annual surveillance. Surgery is the most effective treatment modality, and the role of adjuvant chemotherapy for postoperative management is unclear. EBV is likely to play an important role in the etiology of IPT-like FDC sarcoma.

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Manuscript source: Unsolicited manuscript

Specialty type: Oncology

Country/Territory of origin: China

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: December 23, 2020
Peer-review started: December 23, 2020
First decision: December 30, 2020
Revised: January 2, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Ho CM
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li JH



Key Words: Inflammatory pseudotumor-like; Follicular dendritic cell sarcoma; Spleen; Liver; Prognosis; Epstein-Barr virus

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Core Tip: We show that inflammatory pseudotumor-like follicular dendritic cell (FDC) sarcoma appears to be mildly aggressive and does require annual surveillance. Surgery is the most effective treatment modality, and the role of adjuvant chemotherapy for postoperative management is unclear. Epstein-Barr virus is likely to play an important role in the etiology of inflammatory pseudotumor-like FDC sarcoma.

Citation: Wu H, Liu P, Xie XR, Chi JS, Li H, Xu CX. Inflammatory pseudotumor-like follicular dendritic cell sarcoma: Literature review of 67 cases. *World J Meta-Anal* 2021; 9(1): 1-11
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/1.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.1

INTRODUCTION

Follicular dendritic cells (FDCs) develop from perivascular precursors of stromal cell origin that are seeded throughout the body. They are centrally located within B cell follicles and act as a bridge between innate and adaptive responses[1]. Monda *et al*[2] first reported 4 cases of tumors derived from FDC in 1986. FDC sarcoma is an uncommon neoplasm that can involve lymph nodes or extranodal sites. It usually has an aggressive course in the abdomen[3]. Cheuk *et al*[4] first characterized inflammatory pseudotumor(IPT)-like FDC sarcoma as an EBV-associated tumor of FDC origin in 2001. The most recent World Health Organization (WHO) classification noted that IPT-like FDC sarcoma appears to be indolent and that data on clinical outcome are limited[5].

In this study, we detail the features of 67 cases with IPT-like FDC sarcoma, providing an overview of the current knowledge about the clinical, pathological, and prognostic characteristics of this disease entity.

LITERATURE REVIEW

We systematically searched PubMed, EMBASE, and MEDLINE databases using the search terms "inflammatory pseudotumor-like" combined with "follicular dendritic cell sarcoma" or "follicular dendritic cell tumor" or "fibroblastic dendritic cell sarcoma" or "fibroblastic dendritic cell tumor" and with the restrictions "English" language and "original article." We collated demographic, clinicopathological and follow-up information.

All statistical analyses were performed using SPSS Statistics 25.0 software (IBM Corporation, Armonk, NY, United States). Survival curves were replotted with Graphpad prism 8.0 software (Graphpad Inc., La Jolla, CA, United States). $P < 0.05$ was considered statistically significant, and variables pertaining to accuracy were calculated with 95% confidence intervals.

PATIENT CHARACTERISTICS

Data on a total of 67 cases of IPT-like FDC sarcoma were retrieved from the literature. Patients and disease characteristics are summarized in Table 1. The median age at initial presentation was 53 years (range: 19-79). Females were more commonly affected than males (n = 42 *vs* 25). Patients were asymptomatic or presented with abdominal distension or pain, sometimes accompanied by systemic symptoms such as fever, fatigue, and weight loss. Other cancers were observed in 6 patients before their diagnosis with IPT-like FDC sarcoma including gastric cancer (n = 3), breast cancer (n = 1), pituitary adenoma, meningioma, and acoustic neuroma (n = 1) and diffuse large

Table 1 Clinical characteristics of patients with inflammatory pseudotumor-like follicular dendritic cell sarcoma

Case No.	Ref.	Sex/age	Site	Maximum diameter in cm	Symptom	Treatment	Time to recurrence in mo	Last follow-up in mo	Outcome
1	Rao *et al*[22]	39/M	Spleen	7.2	Asymptomatic	Surgery	NA	NA	NA
2	Zhao[23]	28/F	Abdomen	9	Skin rash, cough	Surgery	NA	NA	NA
3	Kazemimood *et al*[12]	53/F	Colon	3	Abdominal discomfort	Surgery	NA	NA	NA
4	Hommel *et al*[24]	77/F	Spleen	12	Left upper quadrant abdominal pain	Surgery	-	4	NED
5	Hang *et al*[25]	57/M	Spleen	2.7	Asymptomatic	Surgery	-	9	NED
6	Granados *et al*[26]	57/F	Liver	13	Abdominal pain, vomiting	Surgery	-	24	NED
7	Kiryu *et al*[27]	56/F	Spleen	4	Asymptomatic	Surgery	-	24	NED
8	Li *et al*[28]	32/F	Liver	3	-	Surgery	-	8	NED
9	Pan *et al*[29]	78/F	Colon	3.9	Abdominal discomfort, bloody stool	Surgery	-	5	NED
10	Ang *et al*[30]	63/F	Liver	13.4	Fever and lethargy	Surgery	-	48	NED
11	Wu *et al*[31]	45/M	Liver	8	Abdominal pain and weight loss	Surgery	-	9	NED
12	Zhang *et al*[32]	19/F	Liver	6	Abdominal discomfort	Surgery	-	12	NED
13	Deng *et al*[33]	67/F	Liver	4	Cough	Surgery	NA	NA	NA
14	Horiguchi *et al*[34]	77/F	Spleen	8.5	Epigastralgia	Surgery	-	36	NED
15	Kitamura *et al*[35]	74/F	Spleen	3.6	Asymptomatic	Surgery	-	24	NED
16	Kim *et al*[36]	76/M	Spleen	3.2	Asymptomatic	Surgery	-	7	NED
17	Kwon[37]	58/F	Spleen	5	Asymptomatic	Surgery	-	24	NED
18	Nishiyama *et al*[38]	73/F	Spleen	8	Asymptomatic	Surgery	-	144	NED
19	Bui *et al*[39]	50/F	Spleen	8.5	Abdominal pain	Surgery	NA	NA	NA
20	Mograbi *et al*[40]	70/F	Spleen, Pancreas	Spleen: 3, Pancreas: 7	Asymptomatic	Surgery	NA	NA	NA
21	Hu *et al*[41]	49/F	Retroperitoneum	5	Asymptomatic	Surgery	Tail of pancreas (60)	60	NED
22	Vardas *et al*[42]	61/M	Spleen	10	Abdominal pain	Surgery	-	12	NED
23	Agaimy *et al*[43]	52/M	Ileum and mesentery	6	Abdominal pain	Surgery	NA	NA	NA
24	Ge *et al*[44]	54/F	Spleen	3.5	Left upper quadrant abdominal pain	Surgery	-	10	NED
25	Ge *et al*[44]	79/M	Spleen	6	Asymptomatic	Surgery	-	18	NED
26	Zhang *et al*[45]	31/F	Liver	2 masses: 3.5, 2.5	Anorexia	Surgery	-	10	NED
27	Zhang *et al*[45]	48/M	Liver and hepatoduodenal ligament lymph node	Liver: 10, Lymph: 3.5	Asymptomatic	Surgery	-	2	NED

28	Ke *et al*[46]	53/M	Colon	1	Chest and back pain	ESD	-	11	NED
29	Ke *et al*[46]	48/M	Colon	4.5	Left lower quadrant pain	Surgery	-	7	NED
30	Li *et al*[47]	64/F	Spleen	7.2	Upper abdominal pain	Surgery	-	8	NED
31	Li *et al*[47]	61/M	Spleen	6.2	Asymptomatic	Surgery	-	16	NED
32	Li *et al*[47]	42/F	Spleen	4	Left-sided flank pain	Surgery	-	9	NED
33	Li *et al*[47]	57/F	Spleen	13.3	Upper abdominal pain	Surgery	Pulmonary (4)	4	LWD
34	Li *et al*[47]	52/M	Spleen	2.9, 3.7	Back pain	Surgery, chemotherapy	Multiple bone (5)	5	LWD
35	Li *et al*[48]	49/F	Spleen	4.7	Asymptomatic	Surgery	-	NA	NA
36	Li *et al*[48]	56/F	Spleen	8	Abdominal pain	Surgery	-	17	NED
37	Li *et al*[48]	38/M	Liver	8.5	Fatigue, anorexia	Surgery	-	11	NED
38	Li *et al*[48]	42/F	Liver	2, 1.7	Abdominal pain	Surgery	-	36	NED
39	Li *et al*[48]	50/M	Spleen and liver	Spleen: 10, Liver: 3, 1.5, 1	Abdominal bloating, fatigue	Surgery	-	17	NED
40	Li *et al*[48]	39/F	Liver	9	Asymptomatic	Surgery, chemotherapy	Liver (12)	84	NED
41	Choe *et al*[19]	64/F	Spleen	5.5	Asymptomatic	Surgery	-	78	NED
42	Choe *et al*[19]	72/F	Spleen	7.2	Asymptomatic	Surgery	-	18	NED
43	Choe *et al*[19]	53/F	Spleen	3.2	Asymptomatic	Surgery	-	13	NED
44	Choe *et al*[19]	76/M	Spleen	3.2	Asymptomatic	Surgery	-	8	NED
45	Choe *et al*[19]	72/M	Spleen	6	Asymptomatic	Surgery	-	18	NED
46	Choe *et al*[19]	75/M	Spleen	3.5	Abdominal pain	Surgery	-	30	NED
47	Chen *et al*[49]	28/F	Liver	6	Abdominal pain, fatigue, anorexia	Surgery	Liver (48)	48	LWD
48	Chen *et al*[49]	39/M	Spleen	7.4	Asymptomatic	Surgery	-	40	NED
49	Chen *et al*[49]	48/M	Liver	23.3	Abdominal pain, fever, fatigue	Surgery	-	23	NED
50	Chen *et al*[49]	65/M	Spleen and liver	Spleen: 22.3, Liver: Multiple masses (largest: 5.8)	Abdominal pain, fever, fatigue, anorexia, weight loss	Surgery	-	2	DOD
51	Chen *et al*[49]	51/M	Spleen	8.5	Weight loss	Surgery	-	19	NED
52	Chen *et al*[49]	68/M	Spleen	2.3	Asymptomatic	Surgery	-	6	NED
53	Chen *et al*[49]	51/F	Spleen	5.3	Epigastric discomfort	Surgery	-	5	NED
54	Chen *et al*[49]	67/M	Spleen	7.5	Asymptomatic	Surgery	-	5	NED
55	Chen *et al*[49]	60/M	Liver	3	Asymptomatic	Surgery	-	3	NED
56	Chen *et al*[49]	52/F	Spleen	0.9	Asymptomatic	Surgery	-	12	NED
57	Cheuk *et al*[4]	19/F	Liver	12	Right upper quadrant pain, weight loss	Surgery	-	40	NED
58	Cheuk *et al*[4]	56/F	Liver	15	Abdominal discomfort	Surgery	Liver (15, 27 ,35)	56	LWD
59	Cheuk *et al*[4]	40/F	Liver	12.5	Abdominal pain, weight loss	Surgery	Intraabdominal (108)	108	LWD
60	Cheuk *et al*[4]	49/F	Liver	4.2	Asymptomatic	Surgery	-	9	NED

61	Cheuk *et al*[4]	37/M	Liver	15	Weight loss	Surgery	-	42	NED
62	Cheuk *et al*[4]	35/F	Liver	20	Abdominal discomfort, fever, weight loss	Surgery	Liver (30, 40), peritoneum and ascending colon (60)	95	DOD
63	Cheuk *et al*[4]	31/F	Liver	15	Abdominal distension, weight loss	Surgery	-	60	NED
64	Cheuk *et al*[4]	58/F	Spleen	22	Abdominal distension	Surgery	-	4	NED
65	Cheuk *et al*[4]	39/F	Spleen	7.5	Weight loss, fever	Surgery	-	2	NED
66	Cheuk *et al*[4]	61/F	Spleen	3.5	Asymptomatic	Surgery	NA	NA	NA
67	Cheuk *et al*[4]	49/F	Peri-pancreas	9.5	Abdominal distension	Surgery	NA	NA	NA

DOD: Dead of disease; ESD: Endoscopic submucosal dissection; LWD: Live with disease; NA: Not available; NED: No evidence of disease.

B-cell lymphoma (n = 1). Five patients (7.5%) had pre-existing or subsequent autoimmune disease including pemphigus and bronchiolitis obliterans (n = 1), IgA nephropathy (n = 1), nephrotic syndrome (n = 1), bronchial asthma (n = 1), and idiopathic thrombocytopenic purpura (n = 1).

DISEASE CHARACTERISTICS

Thirty-five patients (52.2%) presented solely with spleen involvement and twenty (29.9%) only with liver. For the remaining patients, lesion sites included the abdomen (n = 1), colon (n = 4), spleen and pancreas (n = 1), retroperitoneum (n = 1), ileum and mesentery (n = 1), liver and hepatoduodenal ligament lymph node (n = 1), spleen and liver (n = 2) and peri-pancreas (n = 1). The average size of tumors at all sites was 7.6 cm, ranging from 0.9 to 23.3 cm (median, 6.2 cm). Bulky tumor (≥ 5 cm) was noted in 43 patients (64.2%). Seven patients presented with more than one lesion. The radiologic findings of IPT-like FDC sarcoma have been described in several cases. The abdominal computed tomography scan showed a well-circumscribed or ill-defined, hypodense mass with weak delayed heterogeneous enhancement after contrast enhancement in the spleen or liver. Some of these lesions reveal irregular areas of nonenhancement related to foci of tumoral necrosis and hemorrhage.

PATHOLOGICAL FEATURES

The pathological features are summarized in Table 2. In all, 40 cases (59.7%) presented with necrosis, 22 (32.8%) with hemorrhage, 23 (34.3%) with noncaseating epithelioid granulomas and 22 (32.8%) with fibrinoid deposits in the blood vessel wall. The neoplastic cells were predominantly positive for the follicular dendritic cell markers CD21(54/65), CD23 (34/47), CD35 (50/55) and CNA.42 (13/13). Tumor cells were usually positive for clusterin (8/11), vimentin (6/6), fascin (9/9), and smooth muscle actin (32/39), and variably positive for S100 (6/23), D2-40 (6/10), epidermal growth factor receptor (3/8), muscle-specific actin (5/9), epithelial membrane antigen (2/8), CD68 (8/16), tubulin beta 3 class III (3/6) andγ-synuclein (2/6). There was no staining for CD117, cytokeratin, caldesmon, activin-like kinase 1 (ALK1), desmin or human herpes virus 8 (HHV8). In 4 cases, the tumor cells resembled Reed-Sternberg cells. The neoplastic cells were consistently associated with EBV, and EBV-encoded small RNA (EBER) was positive in 60 cases (95.2%) by *in situ* hybridization. Immunoreactivity for EBV-encoded latent membrane protein 1 (LMP1) was present in 28 cases (82.4%). One case present with *LMP1* gene fragment deletions and point mutations. Increased IgG4-postive plasma cells were found in 6 cases. Mitoses were very rare in most cases and the Ki-67 proliferation index ranged from 3% to 30%.

Table 2 Pathological features of inflammatory pseudotumor-like follicular dendritic cell sarcoma

Markers	Number of cases positive/number of cases tested	Cases positive, %
Necrosis	40/67	59.7
Hemorrhage	22/67	32.8
Granuloma	23/67	34.3
Fibrinoid deposits in blood vessel wall	22/67	32.8
CD1a	0/6	0
CD3	0/7	0
CD20	1/9	11.1
CD21	54/65	83.1
CD23	34/47	72.3
CD30	1/27	3.7
CD31	1/8	12.5
CD34	5/15	33.3
CD35	50/55	90.9
CD68	8/16	50
CD117	0/9	0
LMP1	28/34	82.4
EBER	60/63	95.2
D2-40	6/10	60
CNA.42	13/13	100
Clusterin	8/11	72.7
EGFR	3/8	37.5
Vimentin	6/6	100
CK-pan	0/9	0
Caldesmon	0/7	0
ALK1	0/42	0
Desmin	0/30	0
Fascin	9/9	100
SMA	32/39	82.1
MSA	5/9	55.5
EMA	2/8	25
S100	6/23	26.1
p53	1/6	16.7
HHV-8	0/7	0
TUBB3	3/6	50
γ-synuclein	2/6	33.3

EMA: Epithelial membrane antigen; HHV-8: Human herpes virus 8; LMP1: Latent membrane protein 1; MSA: Muscle-specific actin; SMA: Smooth muscle actin; TUBB3: Tubulin beta 3 class III.

TREATMENT INFORMATION

Information on treatment was available for all 67 patients, most of whom (65/67) underwent surgery alone. Only 2 patients received surgery and chemotherapy (one with two cycles of cyclophosphamide, doxorubicin, vincristine and prednisone, and the other with CHOP-based chemotherapy).

PATIENT OUTCOMES

Disease status at the time of last follow-up was known for 57 of the 67 patients; these patients were eligible for progression-free survival (PFS) analyses. The follow-up time ranged from 2 to 144 mo (mean, 22 mo; median, 12 mo). Overall, 9 of the 57 (15.8%) had local and/or distant recurrence after initial treatment. Two patients died due to disease progression, but two others had no evidence of disease after undergoing a second surgery. The 1- and 5-year PFS for the entire group was 91.5% and 56.1%, respectively (Figure 1). Young age, male sex, and large tumor size may contribute to less disease progression. However, Kaplan-Meier and multivariate analyses failed to confirm that age, sex, tumor size or and pathological features are risk factors for disease progression (Table 3).

DISCUSSION

In 1986 Monda *et al*[2] first reported 4 cases of tumors derived from FDC and in 2001 Cheuk *et al*[4] reported that IPT-like FDC sarcoma were EBV-associated tumors of FDC origin. Currently, FDC sarcomas are classified into conventional and IPT-like FDC sarcoma[5]. Our study indicated that IPT-like FDC sarcoma occurs predominantly in middle-aged adults, with a marked female predilection unlike the equal distribution between the sexes of conventional FDC sarcoma. IPT-like FDC sarcoma typically involves the spleen and/or liver. Genetic lineage-tracing approaches show that FDC develop from splenopancreatic embryonic mesenchymal cells of the Nkx2-5(+) Islet1(+) lineage[6]. FDC emerge from perivascular precursors, engaging B cells in germinal centers of secondary lymphoid organs[1,7]. Our analysis indicated that 7.7% patients had pre-existing or subsequent autoimmune disease. Conventional FDC sarcoma also showed a trend for occurring coincidentally with autoimmune disease[3]. FDC can retain antigens to establish contact with B cells, and secrete milk-fat globule epidermal growth factor 8, which controls the engulfment of apoptotic B cells by macrophages. Excessive accumulation of apoptotic B cells may result in lupus-like autoimmunity[8,9]. Furthermore, FDC was shown to be important for the retention of self-antigen-containing immune complexes in a spontaneous arthritis model[10].

We found that the average size of tumors was 7.6 cm, ranging from 0.9 to 23.3 cm (median, 6.2 cm). Bulky tumor (≥ 5 cm) was noted in 43 patients. Necrosis, hemorrhage, noncaseating epithelioid granulomas, and fibrinoid deposits in blood vessel walls are often present in IPT-like FDC sarcoma. A pooled analysis demonstrated that young age, large tumor size and coagulative necrosis may predict poor prognosis in conventional FDC sarcoma[11], but our data failed to demonstrate that age, large tumor size, and necrosis are risk factors for disease progression in IPT-like FDC sarcoma. The neoplastic spindle-shaped cells of the latter are present within a prominent lymphoplasmacytic infiltrate and may even resemble Reed-Sternberg cells[4,12]. IPTs can also be found in the spleen and liver, and tend to appear as plump spindle cells in a polymorphic inflammatory cell infiltrate[13,14]. IPT-like FDC sarcomas are predominantly positive for the FDC markers CD21, CD23, CD35 and CNA.42, thus distinguishing them from Hodgkin lymphoma and IPTs. Moreover, IPT-like FDC sarcomas are negative for cytokeratin, CD117, caldesmon, ALK1, desmin and HHV8, which can help in the differential diagnosis of epithelial carcinoma, gastrointestinal stromal tumor, inflammatory myofibroblastic tumor, anaplastic large cell lymphoma, rhabdomyosarcoma and Kaposi sarcoma. IPT-like FDC sarcomas are usually positive for clusterin, vimentin and fascin. Strong clusterin staining appears to be a highly sensitive marker of FDC sarcoma. Vimentin staining may confirm mesenchymal origin, but is relatively nonspecific. Fascin staining was not specific for spindle cell tumors and thus does not imply a dendritic cell lineage[15,16].

Our study concluded that 95.2% of patients possessed tumor cells positive for EBER by *in situ* hybridization, and 82.4% were immunoreactive for EBV-encoded LMP1. EBV has a long history of coevolution with humans. The result is a finely balanced

Table 3 Factors evaluated by univariate and multivariate for association with progression-free survival

Variable	Univariate analysis		Multivariate analysis	
	P value	HR (95%CI)	*P* value	HR (95%CI)
Age ≤ 40 yr	0.50	1.55 (0.39-6.16)	0.48	1.78 (0.36-8.77)
Gender, male/female	0.97	0.97 (0.21-4.64)	0.67	1.51 (0.23-10.04)
Tumor size < 5 cm	0.59	0.59 (0.11-3.32)	0.43	0.38 (0.04-4.10)
Necrosis	0.31	0.51 (0.10-2.59)	0.24	0.25 (0.03-2.49)
Hemorrhage	0.99	0.99 (0.27-3.70)	0.84	1.25 (0.13-11.91)
Granuloma	0.58	0.66 (0.16-2.71)	0.85	0.84 (0.14-5.15)

HR: Hazard ratio; CI: Confidence interval.

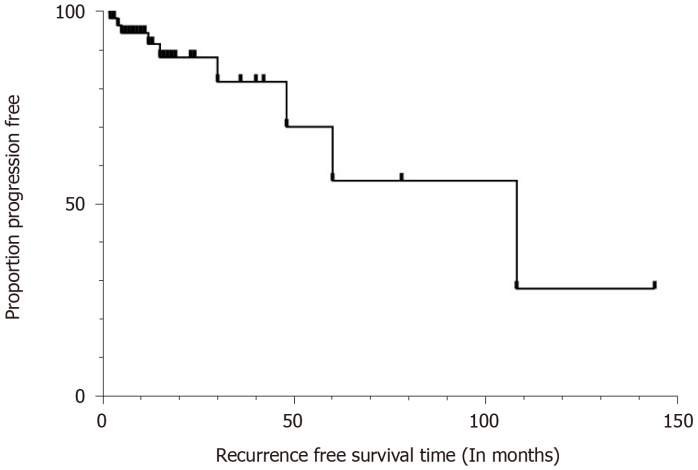


Figure 1 Progression-free survival curve for patients with inflammatory pseudotumor-like follicular dendritic cell sarcoma.

relationship that usually allows the virus to be carried as a lifelong asymptomatic infection. However, the pathogenic potential of EBV has been confirmed in several autoimmune disorders, particularly multiple sclerosis, and a wide range of human tumors[17]. Approximately 2.2 million cases of cancers in 2018 were attributable to infectious agents. It is estimated that EBV accounts for more than 150000 of these, mainly nasopharyngeal carcinomas, Hodgkin lymphomas and Burkitt lymphomas[18]. IPT-like FDC sarcoma cells were consistently positive for EBER and LMP1. Interestingly, increased IgG4-postive plasma cells were found in 6 cases[19]. There is evidence for the presence of increased numbers of EBV-infected cells in IgG4-related lymphadenopathy[20]. EBV lytic reactivation induces IgG4 production in Graves' disease[21]. The etiology of EBV in IPT-like FDC sarcoma needs further research.

Surgery is the most effective therapy for IPT-like FDC sarcoma, and the number of cases treated by adjuvant chemotherapy is limited. Nevertheless, here we saw that 15.8% patients had local and/or distant recurrence after initial treatment. The WHO has noted that data on clinical outcomes are limited, but that the tumor appears to be indolent[5]. Our research revealed that 1- and 5-year PFS for the entire group was 91.5% and 56.1%, respectively.

CONCLUSION

There is a certain risk of relapse after the initial therapy of IPT-like FDC sarcoma, which therefore needs annual surveillance. Surgery is the most effective treatment modality, and the role of adjuvant chemotherapy for the management of postoperative is not clear. EBV plays an important role in the etiology of IPT-like FDC sarcoma.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 February 28; 9(1): 12-28

DOI: 10.13105/wjma.v9.i1.12

ISSN 2308-3840 (online)

REVIEW

Exosomes: A new frontier under the spotlight for diagnosis and treatment of gastrointestinal diseases

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

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Manuscript source: Invited manuscript

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Abstract

Exosomes are small plasma membrane-bound multivesicular bodies ranging in size from 20-100 nm. Exosomes are degraded fragments of mRNA, microRNA, and enriched in proteins, lipids, and nucleic acid. They are produced in the endosomes of most eukaryotic cells and once secreted, exosomes are involved in cell to cell communication and remodeling of the matrix in the extracellular compartment. Exosome biogenesis plays a crucial role in cellular development, inflammation, immunity, hemostasis, carcinogenesis, and degeneration. Due to their unique biochemical and biophysical properties, exosomes serve a variety of functions including biomarkers of diagnostic and prognostic significance. Besides, there is an increasing level of evidence to expand our understanding of the exosomes as novel therapeutic agents. Inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis, hepatic fibrosis, and gastrointestinal malignancies such as colorectal cancer are the potential avenues where exosomes can be applied as cell therapy and immunotherapy and have shown promising results in several in-vitro and animal models. The purpose of this review article is to highlight the emerging role of exosomes as the diagnostic and therapeutic tool in various diseases involving the gastrointestinal tract like IBD, hepatocellular carcinoma, and colon cancer. A thorough literature search was performed on databases such as PubMed, Ovid Medline, and EMBASE to achieve the objectives of this review article.

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: United States

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D, D
Grade E (Poor): 0

Received: October 21, 2020
Peer-review started: October 21, 2020
First decision: November 3, 2020
Revised: December 15, 2020
Accepted: February 22, 2021
Article in press: February 22, 2021
Published online: February 28, 2021

P-Reviewer: Rostami-Nejad M, Zhao CF
S-Editor: Wang JL
L-Editor: A
P-Editor: Li X



Key Words: Exosomes; Gastrointestinal diseases; Inflammatory bowel disease; Colorectal cancer; Hepatocellular carcinoma

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Core Tip: Exosomes are tiny membrane-bound extracellular vesicles that are becoming increasingly popular due to their potential role in regulating various intracellular and extracellular signaling pathways. Exosome biogenesis has been shown to plays a role in cellular development, inflammation, immunity, hemostasis, carcinogenesis, and degeneration. Due to their unique properties, exosomes have the potential to serve as diagnostic, prognostic, and therapeutic vectors. This review provides a concise summary of the role of exosomes as the diagnostic and therapeutic tool in various diseases involving the gastrointestinal tract.

Citation: Naseer M, Hadi S, Syed A, Safdari A, Tahan V. Exosomes: A new frontier under the spotlight for diagnosis and treatment of gastrointestinal diseases. *World J Meta-Anal* 2021; 9(1): 12-28
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/12.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.12

INTRODUCTION

Most eukaryotic cells can internalize portions of their intracellular components to create endosomes. These endosomes then serve as a recycling plant for the cells[1]. However, some endosomes can internalize parts of their membranes to create multivesicular bodies. Vesicles found within the endosomes are called intraluminal endosomal vesicles. If these multivesicular bodies fuse with the plasma membrane releasing their intraluminal content extracellularly, then those vesicles are referred to as exosomes[2,3]. The size of the parent multivesicular bodies ultimately determine the size of the exosomes, which can range between 20-100 nm[4]. Although poorly understood at the time, exosomes first made their appearance in literature as early as the 1940s[5]. In the 1970s these extracellular "membrane fragments" were considered a universal feature of viable cells. In the early 1980s exosomes were considered nothing more than cellular debris that budded directly from the plasma membrane[6]. In 1987 exosomes were given their name by Rose Johnstone who able to isolate endosome-derived vesicles proving their intracellular origin[7]. The next major advancement in the history of exosomes was the discovery of mRNA, microRNA (miR), and proteins found within them[8,9]. Exosome protein composition can be divided into two categories. The first category depends on the endosome from which they originated and contains components from the Golgi apparatus, endoplasmic reticulum, nucleus, and mitochondria. The second category consists of evolutionarily conserved protein molecules that have the potential to serve as a biomarker (Figure 1). Exosomes also contain mRNA or miR which have been shown to alter gene expression in target cells[8]. This has led to increased research in the field and exosomes have now been shown to patriciate in biological processes including signaling, inflammation, waste management, immune response, coagulation, cell death, angiogenesis, neurodegenerative diseases, and cancer[10,11]. Exosomes also play a role in altering the microenvironment of tumor cells to help facilitate angiogenesis and metastasis to a more favorable environment in the setting of hypoxia[12]. Multiple studies have demonstrated the potential for exosomes to be used as novel biomarkers and therapeutic tools in combating cancer. Current research is focused on bringing exosomes to the clinical arena as said biomarkers and therapeutic vectors[13]. The purpose of this review article is to highlight the emerging role in the diagnostic and therapeutic utility of exosomes in various cancerous and non-cancerous diseases involving the gastrointestinal tract.

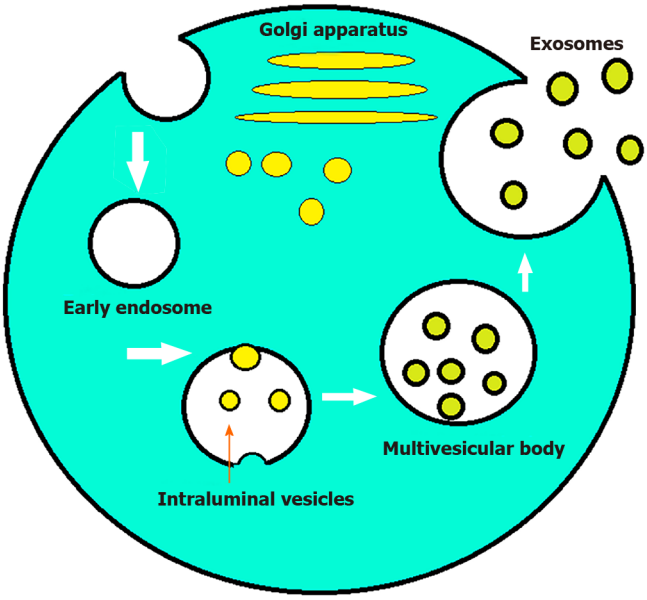


Figure 1 Formation of exosomes. Early endosomes are produced in the endosomal compartments of cells though inward budding of the cell membrane. Multivesicular body (MVB) are formed when the early endosome buds inward into the endosomal lumen creating intraluminal vesicles (ILVs). If the MVB fuses with the cell surface (the plasma membrane), these ILVs are released as exosomes.

ROLE OF EXOSOME IN NON-CANCEROUS GASTROINTESTINAL DISEASES

Exosomes in inflammatory bowel disease

Intestinal epithelial cells interact daily with numerous dietary-derived antigens and bacteria without eliciting immune reactions. Yet, when enteric pathogens are introduced, the immune system activates to fight the novel insult. Homeostasis depends on the ability of the immune system to distinguish normal intraluminal content from invading pathogens. The failure of this process results in inflammatory bowel diseases[14-16]. Although the exact etiology of the disease remains debated, a combination of factors including genetic, immunoregulatory, and environmental likely play a major role[17]. Inflammatory bowel disease (IBD) is a systemic, chronic, noninfectious, and relapsing inflammatory disorder of the gastrointestinal tract consisting primarily of Crohn's disease and ulcerative colitis. The symptoms of the disease commonly include abdominal pain, bloody diarrhea, weight loss, and fever[18,19]. Current therapy for the disease focuses primarily on immunosuppression with agents such as anti-tumor necrosis factor (TNF) monoclonal antibodies, immunomodulators, anti-inflammatory drugs, and thiopurine agents[20]. The incidence of IBD is increasing in developed countries and the United States alone one to 1.5 million people estimated to be affected by the disease[21-23]. Patients with the disease experience a reduction in their overall quality of life and increased mortality primarily due to an increased risk for colitis associated colorectal cancer (CRC)[24-26]. Current therapies for IBD have their limitations and many patients are unable to achieve remission. As such, novel therapies ranging from the use of herbal extracts to the introduction of harmless parasites have been proposed[27-29]. One potential avenue worth exploring in the treatment of IBD is the use of exosomes as novel biomarkers and therapeutic vectors.

Currently, the gold standard for diagnosing IBD is ileocolonoscopy. Endoscopic visualization is necessary due to the discrepancy between clinical symptoms and the extent of the disease. The endoscopic assessment provides critical information about the severity of the disease and aids in decision making regarding medical management. However, colonoscopies are associated with increased cost and patient discomfort. As such development of less invasive tools for diagnosing IBD are necessary.

Exosomes show promise as novel biomarkers for diagnosing IBD and assessing its progression (Table 1). One such biomarker is proteasomes subunit alpha type 7 (PSMA7) which is markedly upregulated in the salivary exosomes of a patient with IBD when compared to healthy individuals[30]. The exact relationship between the

Table 1 Exosomes as promising novel biomarkers for diagnosis of inflammatory bowel disease and its progression

Exosomal component	Origins	Effects and mechanisms	Significance	Exosome structure effects
Baseline proteins in regular controls[16]	Intestinal epithelial cells	TGF-β1-dependent immunosuppression of T cells and dendritic cells	Treatment	Localization in intestinal tract and epithelial cell adhesion
PSMA7[30]	Saliva obtained from IBD patients	Higher expression in IBD patients when compared to normal	Diagnosis	Long distance transportation and protection
Annexin-1[31]	Serum of dextran sulfate sodium induced colitis in mice	Protection of epithelial cells anti-inflammatory	Diagnosis/treatment	Annexin-1 efficiency enhancement
Inflammatory proteins and mRNA[32]	Patients with IBD with a high endoscopic score (≥ 1)	Increased translation of IL-8 protein	Diagnosis/treatment	Membrane carrier
Acute phase proteins in IBD[34]	Serum of dextran sulfate sodium induced colitis in mice	Phosphorylation of p38 and ERK and production of tumor necrosis factor α resulting in macrophage activation	Diagnosis/treatment	Membrane carrier

IBD: Inflammatory bowel disease; PSMA7: Proteasomes subunit alpha type 7; IL-8: Interleukin 8; TGF: Transforming growth factor.

development of IBD and salivary exosomal PSMA7 has yet to be elucidated, but the ease with which they can be collected may represent a better diagnostic approach towards IBD[30]. Another potential biomarker for diagnosing IBD is annexin-1 containing exosomes. These exosomes arise from the intestinal epithelial cell and are overexpressed during inflammatory responses. Annexin-1 has been shown to play a role in the healing process that occurs in intestinal epithelial cells following mucosal damage. Patients with IBD have shown have increased levels of annexin-1 containing exosomes making them a potential new biomarker for diagnosing IBD[31].

The pathophysiology of IBD involves a variety of factors including environmental factors, genetic factors, microbial dysbiosis, and immune system dysregulation. Current treatment for IBD centers on maintaining immuno-tolerance to obtain remission. Current research suggests that immune tolerance may be established through the regulation of activated T cells. Exosomes have been shown to have an immunosuppressive activity that can down-regulate T cells resulting in decreased severity of the disease[16]. Although the mechanism is not fully understood, the transfer of intestinal exosomes from healthy mice into those with IBD has been shown to decrease the severity of IBD[16]. On the other hand, the transfer of exosomes from IBD patients into human colonocytes cell line DLD-1 has been shown to increase the expression of inflammatory markers such as interleukin (IL)-8 within the cell line[32]. Another way that immune tolerance may be established is through the suppression of macrophages. The activation of macrophages has been suggested to play a critical role in the pathogenesis of IBD[33]. Exosomes have been shown to play a role in intercellular communication and can thus modulate the macrophage-mediated immune response[34]. Inducing acute colitis in mice results in the exosomal expression of 56 proteins to change when compared to healthy controls[35]. Most of the proteins that are expressed are acute phase immunoglobulins and proteins which participate in the coagulation cascade and the complement pathway[36]. As such exosomes represent excellent targets for blocking the activation of macrophages.

Exosomes in hepatic fibrosis and cirrhosis

Chronic liver injury and inflammation result in the production of insoluble collagen fibers and extracellular matrix components resulting in fibrosis and eventually cirrhosis. Exosomes have been shown to participate in this process and thus can serve as potential biomarkers and therapeutic vectors[37]. The gold standard for diagnosis of liver fibrosis and cirrhosis is a biopsy. This is an invasive and expensive procedure the carries the risk of infection, internal bleeding, and injury to the biliary tract. Early research in exosomes shows promise in detecting fibrosis and cirrhosis. Exosomes collected from the serum of a patient with chronic hepatitis C (HCV) have been associated with inflammation and severity of fibrosis when enriched with CD81[38]. Human patients with F3-4 fibrosis were also noticed to have decreased levels of miR-34c, miR-151-3p, miR-483-5p, or miR-532-5p in their serum exosomes. These same miRNAs were also decreased in the serum exosomes of mice with carbon

tetrachloride-induced liver fibrosis[39]. Although promising, more research is needed before exosomes can be used in a clinical setting. However, the potential of exosomes and the ease in which they can be collected has the promise of reducing invasive procedures in the future for diagnosing fibrosis or cirrhosis.

Exosomes in acute and chronic pancreatitis

Emerging research shows that exosomes play a role in pancreatic diseases including acute pancreatitis, chronic pancreatitis, and pancreatic cancer. In acute pancreatitis exosomes have been shown to participate in pulmonary dysfunction by penetrating the alveolar endothelial barrier and activating macrophages by changing their phenotype from M2 to M1 resulting in injury to the lungs[40]. In pancreatic cancer exosomes derived from pancreatic ductal adenocarcinoma are internalized by Kupffer cells and cause secretion of transforming growth factor (TGF)-β. This results in increased fibronectin production by hepatic stellate cells resulting in activation of fibrotic pathways and proinflammatory milieu to facilitate tumor cell metastasis[41]. Exosomes have been shown to participate in complex signal pathways and such have a potential to serve as novel biomarkers when it comes to pancreatic diseases. In the case of pancreatic cancer, the first presentation are the clinical symptoms and the workup to confirm the diagnosis is costly and invasive in the form to computed tomography scan, endoscopic ultrasound, and biopsy. The current biomarker for pancreatic cancer is carbohydrate antigen 19-9 (CA19-9) which has a sensitivity and specificity of 79%-81% and 82%-90% respectively[42]. However, this is only the case in which patients are suspected to have symptoms of pancreatic cancer. When it comes to its function as a screening maker it has a low positive predictive value (0.5%-0.9%)[42]. As a result of this, better biomarker is necessary, and exosomes have the potential to fill this role.

Most of the research concerning the use of exosomes as biomarkers is in its early stages and more rigorous clinical studies are needed before they play a role in the clinical arena. However, two candidates merit further attention. One is the combination of proteoglycan glypican-1 and circulating exosomes. These exosomal markers are found to be significantly elevated in patients with pancreatic ductal adenocarcinoma when compared to healthy control[43]. Furthermore, these markers could discriminate between patients with each stage of pancreatic cancer (carcinoma *in situ*, stage I as well as stages II-IV) from patients with benign pancreatic disease with a 100% sensitivity and specificity[43]. Second is a recent study which compared exosomal proteoglycan glypican-1 and CA19-9 to high exosomal levels of miR-10b, miR-21, miR-30c, and miR-181a and low miR-let7a. This study found the exosomal miRNAs mentioned could not only differentiate pancreatic ductal adenocarcinoma patients from healthy controls, but also those with chronic pancreatitis. Furthermore, these exosomal levels decreased to normal values 24 h following resection whereas the levels of proteoglycan glypican-1 remained elevated[44]. Exosomes can serve as a frontier for much needed novel biomarkers in the arena of pancreatic diseases and the early research have been promising. With further studies these markers can soon have a role to play in the clinical setting as well.

Exosomes in gastritis

Chronic gastritis occurs when the stomach lining becomes inflamed. This can be due to various factors including helicobacter pylori, ethanol use, autoimmune diseases, chronic stress, or certain medication. Currently this disease is diagnosed clinically or with the aid of endoscopy. Untreated chronic atrophic gastritis has the potential for evolving into gastric cancer. Exosomes have been suggested as novel biomarkers for the detection of chronic gastritis. Currently more research is needed in this field, but early studies suggest that exosomes play a role in the development of chronic gastritis. In one study, exomes from chronic gastritis patients with *Helicobacter pylori* (*H. pylori*) infection were shown to up regulate the expression of a proinflammatory cytokine (IL-1α) *via* IL-6 trans-signaling[45]. The proinflammatory effect that results due to the influence of IL-1α is a known initiator of inflammation seen in human diseases[46]. In another study, the exosomal miRNA of patients with chronic atrophic gastritis was compared to those with chronic non-atrophic gastritis. The results suggested that hsa-miR-122-5p is significantly upregulated compared to other components and has the potential to serve as a biomarker for chronic atrophic gastritis[47]. Exosomes also have been shown to play a role in the protective effect seen with *H. pylori* infection in inflammatory bowel disease. Exosomes derived from *H. pylori* infected patient were shown to promote NLRP12 expression *in vitro* human intestinal epithelial cells. NLRP12 was then shown to inhibit the Notch signaling pathway resulting in a decrease in chemokine MCP-1 and MIP-1α. These exosomes where then injected into dextran sulfate sodium induced colitis mice which had an improvement in colitis

symptoms associated with an increase in NLRP12[48]. Currently the studies looking at the relationship between exosomes and chronic gastritis are ongoing. Early data shows that exosomes do play a role a role in gastritis, but further studies are needed to figure out the nature of this interaction.

Exosomes in viral hepatitis

Exosomes have been known to participate in complex cellular communication. One way in which they accomplish this is by transfer genetic material between cells. This normal process, however, can also be hijacked by viruses to promote proliferation and avoid immune response. In a study by Lin *et al*[49] the exosomes found within the serum of patients with chronic hepatitis B (HBV) infection contained HBV proteins and nucleic acids. Furthermore, these components were transferred to hepatocytes in an active manner. Natural killer cells from healthy donors and those with chronic HBV infection were shown to interact with and uptake exosomes containing HBV nucleic acids[49]. These exosomes then impaired natural killer cells resulting in dysfunction[49]. Similarly, patients infected with HCV were also found to have HCV viral RNA and proteins within their exosomes[50]. Exosomes that contained HCV were shown to increase the infectivity of HCV compared to exosomes free of HCV[51]. Also, exosomes helped HCV escape immune response up upregulating Gal-9 which is associated with T-cell inhibition[52]. Due to the role exosomes play in propagating infection by HBV and HCV, they also have the potential to serve as novel therapeutic targets. More research is needed to further understand the nature of exosome use by viruses and development of therapies.

ROLE OF EXOSOME IN CANCEROUS GASTROINTESTINAL DISEASES

Exosomes in CRC

Emerging evidence is beginning to show the potential for exosomes in the disease process of CRC. Due to its resistance to treatment and metastatic properties, CRC is the third leading cause of cancer mortality worldwide[53]. CRC has a high prevalence in both men and women and carries a low 5-year survival, especially when it has progressed to stage IV[54]. Of those newly diagnosed with CRC, approximately 25% have distant metastases at the time of presentation[55,56]. Furthermore, an additional 25% will develop distant metastases two years after the initial diagnosis. The prevalence of metastasis observed in CRC serves as a major challenge in its treatment. CRC is known to preferentially metastasize to the liver and a lesser extent the brain, bones, and lungs[57,58]. Exosomes from cancer cells have been shown to preferentially fuse at predicted sites of their metastasis[59]. The unique integrin expression pattern is thought to be the cause of this observed effect[59]. As exosomes likely play a role in the metastasis of CRC; they have the potential to serve as targets for therapeutic interventions. Exosomes also have the potential to serve as novel biomarkers for detecting and monitoring the progression of the disease.

Cancers of different types are known to be associated with their specific exosomal membrane and cytosolic proteins. These proteins can serve as potential biomarkers for detecting CRC in patients. In the exosomes of a patient with CRC different proteins are upregulated when compared to healthy controls[60]. An interaction between FN1 and matrix metalloproteinase 9, both of which are upregulated in patients with CRC, has been shown to cause changes in tumor microenvironment favoring progression and metastasis by integrin signaling and cytoskeletal reorganization[60]. Another potential biomarker for the diagnosis of CRC is a heat shock protein 60 (Hsp60). This protein is known to play a pivotal role in tumorigenesis and is upregulated in exosomes of patients with CRC. Hsp60 can also be used to monitor response to treatment as its level return to normal after resection of CRC tumor[61].

Exosomes also carry miRNAs which are known to play an important role in the progression of CRC. The expression of these molecules differs in CRC when compared to healthy controls. As such, miRNAs can serve as reliable biomarkers for the diagnosis and staging of CRC. Seven of these molecules (let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, and miR-1246) are known to be upregulated even in the early stages of CRC. These markers can also be used to monitor treatment response, as their levels in the serum drop after tumor resection[62]. Another potential biomarker that can help in the staging of CRC is miR-193a. Higher levels of circulating miR-193a are associated with advanced stages of CRC, whereas lower levels are seen in the early stages and healthy controls[63]. In addition, miRNAs can be used to monitor for the recurrence of CRC as higher levels of miR-17-92a after therapy correlates with

recurrence. The difference in expression of miRNA can also be used as a prognostic tool, as a higher expression of miRNA-19a is associated with a poorer prognosis when compared to those with low expression[64].

Through their role in altering the tumor microenvironment, exosomes have been shown to impact tumor initiation, progression, chemoresistance, and even metastasis. The precise mechanism by which they accomplish this has not been delineated. As such, more research is needed before exosomes can play a central role as novel biomarkers. Although the use of exosomes as biomarker show promise in research done on cell lines, animal models, and human body fluids; large randomized clinical trials are still needed for validation. Moreover, further studies comparing the existing modalities and exosomes are still needed to show if exosomal biomarkers allow for earlier detection of CRC.

Research focusing on strengthening host immunity to combat CRC *via* immunotherapy shows promise. Dendritic cells (DCs) are potent antigen-presenting cells that play an important role in regulating both the adaptive and innate immune responses[65]. Dendritic cell-derived exosomes (Dex) are exosomes that are secreted by DCs and have been shown to elicit a strong immune response against cancer cells[50]. This response is due to an abundance of immunostimulatory membrane proteins being present on the surface of Dex (integrins, CD81, CD63, MHC class I and II)[66,67]. In mouse models, Dex loaded with tumor peptides can invoke a stronger immune response in cytotoxic T cells than even tumor peptide loaded DCs themselves[68]. Exosomes produced by cancer cells inhibit the proliferation and cytotoxic effects of natural killer cells[69]. Furthermore, they carry the Fas ligand on their surface to induce T cell apoptosis[70]. In contrast, exosomes produced by DCs activate anti-tumor immunity and cause tumor cell lysis[71,72]. Dex loaded with tumor antigens have already been tested in clinical trials and were found to be safe and effective in activating antitumor immunity in the cases of melanoma and non-small cell lung cancer[73,74]. While the use of Dex in the treatment of CRC remains to be further studied, phase I clinical trials have already begun. In the cases of advanced CRC, the combination of ascites-derived exosomes and granulocyte-macrophage colony-stimulating factor has been shown to induce the activation of antitumor cytotoxic T cells. Treatment with this immunotherapy was not only safe but also prolonged the survival of patients with CRC in the metastatic stage of the disease[75]. While the use of Dex in the treatment of CRC shows great promise, its success has only been observed in a few small trials. Large randomized clinical trials are still needed before Dex can play a role in the clinical arena. Furthermore, research is still needed to modify Dex to elicit a stronger immune response from innate host immunity.

Exosomes play an important role in the progression of CRC and thus serve as excellent targets for therapeutic interventions. They have the potential to serve as delivery vectors for chemotherapeutic agents. Since exosomes have a slower rate of clearance from the bloodstream, they are superior to existing modalities such as liposomes. They also boast the advantage of a greater degree of specificity for CRC cells. Exosomes can also serve to enhance host immunity against cancer cells as shown by dendritic cell-derived exosomes. They could also be used to enhance chemosensitivity in cancer cells through the use of miRNA, increasing the efficacy of chemotherapeutic agents. While large clinical trials and translational studies are still needed to validate the use of exosomes in a clinical setting; they represent promising therapeutic opportunities in the fight against CRC.

Exosomes in hepatocellular carcinoma

The incidence of hepatocellular carcinoma (HCC), one of the most frequently diagnosed cancer, is increasing worldwide[76-78]. Increased technological development and the availability of novel screening methods are likely contributing factors; but despite advancements in screening and prevention mortality rates continue to increase[79,80]. Current screening and diagnostic tools are limited to abdominal ultrasound, computed tomography scans, magnetic resonance imagines, liver biopsies, and surveillance by alpha-fetoprotein (AFP) levels[81]. Although AFP levels > 400 ng/mL are highly correlated with HCC, only 30% of patients with HCC present with elevated AFP levels[81]. As a result, early-stage tumors are often missed causing patients to present with advanced stages of the diseases; less than 30% of these cases are amenable to surgical intervention[82-85]. As such, more sensitive methods are needed to detect these tumors. Exosomes have the potential to not only offer new tools for detection and surveillance but also open new avenues in the treatment of HCC.

The intercellular communication *via* exosomes has been linked with helping cancer cells create a microenvironment that serves to induce angiogenesis, avoid host cell immune response, interact with the extracellular matrix, create a pre-metastatic niche,

and even develop resistance to chemotherapeutic agents[86-91]. As such, the information contained within exosomes reflects the real-time status of cancer cells. Analysis of this data can help in cancer detection, determining the stage of cancer, its recurrence, and response to chemotherapy. This information presents itself in the form of miR or protein biomarkers that have been concentrated within the exosomes which would otherwise go undetected in serum[92].

miRNA expression varies based on conditions and diseases affecting hepatocytes. Patients with HCC have higher exosomal levels of miR-18a, miR-221, miR-222, and miR224 when compared with those who have chronic hepatitis B or cirrhosis without HCC[75]. A strong correlation was also noticed between miR-21 and patients with chronic HBV[92]. A subsequent study confirmed that high levels of miR-21 were correlated with the advanced tumor stage. Patients with HCC had higher levels of miR-21 when compared to those with chronic HBV or healthy volunteers[93]. Certain miRs (939, 595, and 519d) have also been shown to be superior to AFP as a diagnostic biomarker based on receiver operating characteristic curve analysis[94].

In a separate study comparing patients with chronic HCV *vs* those with HCC secondary to HCV, patients with HCC had significantly lower miR-16 Levels. Moreover, miR-16 could be used to discriminate HCC from chronic HCV with a sensitivity of 57.5% and specificity of 70%. When miR-16 was combined with AFP, the sensitivity of the test improved to 85%, and the diagnostic accuracy improved to 87.5%. Additional associations were also noticed between miR-16 and the number of HCC tumors and their size[95]. In rat models, hepatocytes were converted into HCC cells *via* diethylnitrosamine. Variation in miR-10b, miR-21, miR-122, and miR-200a expression gradually began to appear with the development of liver disease and achieved the greatest change as cells transitioned to cirrhosis and HCC. In comparison, AFP levels remained unchanged until the early stages of HCC[96].

These studies demonstrate that exosomes directed research continues to progress in the area of primary liver disease. Current trends indicated that exosomes may have a role to play in the surveillance and diagnosis of HCC in the future clinical setting. However, further studies are needed to find reliable exosomal biomarkers and more work is needed in developing technologies to extract and accurately test the samples obtained.

Although the mechanism of resistance is not yet fully understood, HCC is known to be highly resistant to chemotherapy. Mounting evidence is showing that exosomes facilitate intercellular communications between HCC cells allowing them to develop resistance to chemotherapeutic agents. One study demonstrated that treatment with TGF-β and sorafenib caused increased expression of long-noncoding RNA within exosomes. Long intergenic non-protein coding RNA, regulator of reprogramming was then shown to enhance the expression of CD133+ tumor-initiating cells resulting in chemoresistance[97]. Another study demonstrated exosomal lincRNA-VLDLR not only promoted cell cycle progression in cancer cells, but also helped to develop resistance by expression of ABCG2 transporter which actively exported chemotherapeutic agents such as sorafenib, camptothecin, and doxorubicin out of the cell[98]. An *in vitro* model demonstrated that exosomes derived from patients with HCC activated the HGF/c-Met/Akt signaling pathway inducing resistance to sorafenib *via* inhibition of sorafenib-induced apoptosis[99].

Exosomes present a novel avenue to explore in the treatment of HCC, as they serve an integral part in cancer cell communication. Vps4A is a protein that weakens cell response to exosomes and thus shows potential as a therapeutic agent for blocking said communication. This molecule is frequently downregulated in HCC and its expression deactivates the PI3K/AKT pathway resulting in decreased growth, migration, and invasion by HCC cells *in vitro*[100]. Exosomes naturally accumulate in the liver after intravenous delivery. This makes them an excellent vehicle for drug delivery; they are also stable, biocompatible, and resistant to proteases or RNases[101]. MiR-355 is a molecule that has shown to have both anti-fibrotic and anti-neoplastic capabilities. In one study, the hepatic stellate cell was used to generate and deliver exosomes containing miR-335 to HCC cells. When this treatment was administered to HCC cells in vitro, inhibition of cell proliferation, and invasion was noted. When tumor-bearing mice were treated, tumor shrinkage was observed[102].

A potential therapeutic option for HCC might lie in strengthening host immunity. Exosomes are efficiently taken up by dendritic cells and can participate in antigen presentation to tumor-specific cytotoxic T cells. In mice exosomes from HCC cells have been used to confirm cross-protection against various tumors with shared antigens independent of the MHC molecules of the donors[103]. In a separate study, a tumor-suppressive effect was noticed when exosomes derived from HCC cells expressed CD9 and CD63 surface markers. These exosome markers are known to induce the cytotoxic

effects of dendritic and NK cells. The activation of this cell-mediated immunity was likely the cause of the observed effect[102]. In a separate study, a significant decrease in tumor growth and prolonged survival rates were observed; when mice with HCC were exposed to exosomes obtained from AFP-expressing dendritic cells. These exosomes stimulated CD8+ T cells, activated interferon gamma, IL-2, IL-10, TGF-β, and inhibited CD25+, Foxp3+, regulatory T cells resulting in a strong antigen-specific immune response. The same response was observed with exosomes obtained from AFP-expressing bone marrow stromal cells[104].

Lastly, exosomes may also have the potential to increase chemosensitivity in cancer cells resulting in increased efficacy of chemotherapeutic agents. MiR-122 has been shown to promote chemosensitivity in HCC cells by down-regulating IGF1-R, metalloprotease 10, and cyclin G1[105]. When HCC cells were treated with miR-122 containing exosomes, they displayed increased sensitivity to sorafenib and 5 fluorouracil[106]. Despite the potential of exosomes, more research is needed before they can play a role in a clinical setting. One significant flaw of exosomes lies in the ability to produce exosomes that contain therapeutic siRNA and active drugs. Preliminary research in the field shows promise, but more work is still needed. Mesenchymal stem cells derived exosomes have been used to pack and deliver therapeutic siRNA and active drugs such as paclitaxel and deserve further investigation[107].

Exosomes in gastric cancer

Gastric cancer (GC) is associated with poor prognosis despite all the recent advancements in chemotherapy, surgical techniques, and radiotherapy[108]. This in part is due to GC presenting in later stages of the disease due to its relatively asymptomatic nature early in its course. Exosomes have been shown to participate in the progression of GC and as such have the potential to serve as novel biomarkers resulting in early diagnosis and increased long-term survival of patients. Examples include exosomes derived from GC cells which have been shown to promote proliferation in an autocrine manner by activation of PI3K/Akt and MAPK/ERK pathways[93]. GC cell exosomes have also been shown to increase proliferation and invasion in an exosome-dependent manner by activation of the MAPK signaling pathway *via* CD 97[109,110].

Potential GC biomarker targets include the BARHL2 gene found within the exosomes in gastric juice. By looking at the methylation level of the gene, GC patients can be discriminated from non-GC control with a sensitivity of 90% and a specificity of 100%. However, this study only contained 20 GC patients and 10 non-GC control and further studies with a larger sample are needed[96]. Other promising biomarkers include exosomal proteins such as GKN1, TGF-β1, and TRIM3. GNK1 Levels are lower in healthy controls when compared to a patient with GC[111]. Increased exosomal TGF-β1 Levels are associated with the advanced stage of GC and increased TRIM3 Levels are associated with GC when compared to healthy controls[112-115]. Other potential biomarkers include exosomal miRNA which has also been shown to not only have diagnostic, but prognostic value as well. Elevated miR-19b-3p and miR-106a-5p levels in serum exosomes are more sensitive and specific for GC than AFP and CA-199[116]. High levels of miR-423-5p in serum exosomes have been correlated with lymph node metastasis and poor prognosis[117]. Post-surgery higher miR-451 Levels predict a significantly poorer prognosis than lower level[118]. Lastly, exosomal lncRNA could also serve as promising new biomarkers. Exosomes found in the plasma of stage I GC patients have significantly upregulated lncUEGC1 when compared to healthy controls. Plasma exosomal lncUEGC1 had a better diagnostic value than CEA in distinguishing stage I GC patients from healthy controls[49]. Overall exosomes show great potential as diagnostic and prognostic biomarkers for GC. However, further research is still needed to test these biomarkers in larger clinical trials to validate preliminary observations.

Exosomes in pancreatic cancer

Globally, pancreatic cancer remained one of the most aggressive malignancy with 5-year relative survival rate as low as 40% in patients with localized disease and 3% in patients with distant metastasis[119]. The poor prognosis is attributed to factors such as lack of early diagnosis, high risk of lymphovascular invasion leading to early distant metastasis, and sub-optimal response to traditional therapies[107]. Exosomes have been identified to play a key role in cellular communication, signaling, and transportation. Due to these important properties, exosomes can serve as potential biomarkers for a better understanding of the tumor behavior and potential for metastasis. A recent study published by Que *et al*[120] highlights the use of pancreatic adenocarcinoma-relate miRNAs (miR-21, miR-155, miR-17-5p, and miR-196a) as potential biomarkers in 49 patients. Compared to controls, patients with advanced or unresectable pancreatic

adenocarcinoma were found to have to higher expression of miR-17-5p and miR-21[108]. However, for localized pancreatic cancer *i.e.* stages I and IIA plasma microsomal exosome miR-196a is found to be elevated in pancreatic ductal adenocarcinoma, and miR-1246 is significantly elevated in patients with intraductal papillary mucinous neoplasms[121]. Certain exosomal proteins are also found to be elevated in a patient with pancreatic cancer. A study published by Melo *et al*[122] indicated that exosomes in a patient with pancreatic cancer are enriched in glypical-1 and the levels were significantly elevated in the cancer group than in controls with sensitivity and specificity of 100%. Other exosomes that were found to be expressed by the metastatic pancreatic cell to liver and lung are macrophage migration inhibitory factor and exosomal integrins such as α6β4 and α6β1[41,43]. In addition, exosomes have a role in the management of pancreatic cancer due to their unique biochemical structure and protein arrangement which enable them to act either as potential therapeutic targets or as nanoparticle carriers to transport proteins and drugs across cell membranes[123].

Exosomes in the cholangiocarcinoma

Cholangiocarcinoma (CCA) is a rare but aggressive malignancy involving the biliary tree. Globally, CCA accounts for 10%-25% of the primary liver cancers which constitute second most common cancer after HCC[124]. Though CCA is more prevalent in Asian countries but in recent years the incidence is even rising in Western world. CCA arises from biliary epithelium and classified as intra-hepatic and extra-hepatic (peri-hilar and distal) subtypes based on location[125]. Chronic inflammatory diseases such as primary sclerosing cholangitis, chronic cholestasis and infections are the common risk factors for CCA. However, there are significant number of cases arising in the absence of known risk factors. The cancer carries poor prognosis and so far, surgical resection is the only curative treatment available with chemoradiation therapy for locally advanced and distantly metastatic cancers[126].

There is substantial evidence that exosomes play important role in the progression of the CCA through regulating tumor microenvironment[127]. CCA microenvironment consists of dense stroma enriched with immune cells and extracellular matrix. Exosomes derived from CCA modulates tumor microenvironment and promotes formation of the tumor stroma by regulating the expression of α-smooth muscle actin mRNA, differentiation of fibroblasts particularly cancer-associated fibroblasts (CAFs) and interleukin-6 by interacting with mesenchymal stem cells[128]. CAFs are known to promote tumorigenesis by producing and releasing tumor growth factors like fibroblast growth factors and vascular endothelial growth factor. There is increasing evidence that CCA-derived exosomes are involved in cell-cell communication and transfer of the oncogenic proteins. Dutta *et al*[129] studied the protein profiles of CCA-derived exosomes and their potential roles in CCA progression. In this study, CCA derived exosomes named KKU-M213 and KKU-100 were incubated with normal human cholangiocyte cells *i.e.* H69 and proteomic analysis was performed. These isolated exosomes were found to be internalized into H69 cells, leading to migration and invasions of these cells by increasing the expression β-catenin and reducing the expression of E-cadherin. In addition, they are involved in metastasis by inhibiting the function of tumor suppressing genes such as p53 and chemoresistance. Furthermore, by inhibiting cytokine-induced killer (CIK) cells exosomes enables tumor cells for immune escape[130]. Like the proteins in exosomes, non-coding RNA such as miRs and circular RNAs are found to be involved in the progression of CCA. Particularly, miR-205-5p and circ-0000284 Levels were associated with invasion and migration of CCA cells in several studies[131,132].

Therapeutic role of exosomes in the management of CCA has been studied well in human and animal models both as a primary target to inhibit tumor progression and drug delivery. Chen *et al*[133] explored the impact of tumor derived exosomes on the antitumor activities of CIK cells. The results of the study showed that by down regulating the population of $CD3^+$, $CD8^+$, NK ($CD56^+$), $CD3^+CD56^+$ cells and decreasing secretion of TNF-α and perforin, RBA derived exosomes inhibit the antitumor activity of CIK cells. In another study conducted by Wang and coauthors, exosomal circ-0000284 found to be a therapeutic target for CCA[134].

CONCLUSION

Exosomal composition changes in response to the state of the cells from which they are released. They provide a view of the physio-pathological process occurring within cells in real-time. Current research indicates that exosomes play a role in modulating

gene expression and cell function. They are also readily available in blood, urine, and saliva marking them as promising candidates as a novel biomarker in the detection of IBD and cancer. An increasing number of studies are expanding our understanding of the potential of exosomes in diagnostic testing. However, a standard method to isolate, analyze, and track exosomes has yet to be developed limiting their application in a clinical setting. Tumorigenesis is a complex process and while certain molecules show promise, further research is still needed to identify biomarkers unique to cancer-derived exosomes. These new biomarkers then need to be tested against current detection and surveillance techniques in large randomized clinical trials. Exosomes have also been shown to play a role in managing the tumor microenvironment, helping cancer cells grow, metastasize, and avoid host immune response. They have also been shown to play a role in the development of cancers such as HCC and CRC. Given the key role they play in the propagation of cancer, they have the potential to serve as novel therapeutic vectors. The biocompatibility of exosomes makes them excellent biocarriers. Many different cell types have been used to engineer exosomes for delivering therapeutic cargos as a means for targeted therapy. The clinical application of current models, however, is limited by inefficiency. As research in the field expands, more efficient methods for generating exosomes will emerge, allowing their transition into the clinical arena. Exosomes carry miRNA and proteins that have been shown to have both a negative and positive effect on cancer cells. These molecules have been shown to limit cancer growth, increase sensitivity to chemotherapy, and activate the immune response resulting in tumor lysis. On the other hand, miRNA and exosomal proteins have also been linked to cancer growth, propagation, and metastasis. Different tumors have different characteristics and likewise, the effect of exosomal cargo on them is equally varied. Further research is needed to characterize these effects so appropriate therapeutic vectors can be selected. Cancer is an adaptive process and more innovation is needed before exosomes can be used for cancer therapy. However, early studies show considerable promise and with further research, exosomes may yet play an increasing role in a clinical setting.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 29-39

DOI: 10.13105/wjma.v9.i1.29

ISSN 2308-3840 (online)

MINIREVIEWS

Biofat grafts as an orthobiologic tool in osteoarthritis: An update and classification proposal

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Author contributions: Purita J wrote the manuscript; Lana JFSD proposed the research subtopics; Fonseca LFD was responsible for navigating the literature and sharing the relevant studies that were included in this review; de Andrade M and Rodrigues LM shared significant knowledge regarding the use of orthobiologics in regenerative medicine; Mosaner T formatted the citations and compiled the references; Centurion P and Purita J revised and formatted the body of the manuscript, verifying spelling, punctuation and grammatical errors; Macedo RDR created Table 1 and was also responsible for reviewing and approving all the modifications made to the manuscript, from draft to final version.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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Abstract

Among degenerative musculoskeletal disorders, osteoarthritis remains one of the main causes of pain and disability in the adult population. Current available alternatives to alleviate symptoms include conservative treatments such as physical therapy, anti-inflammatory drugs and an educational approach to lifestyle modification. The use of certain analgesics, such as opiates and corticosteroids offer short-term results but does not address the etiological source of pain and disability. In addition, prolonged use of such medications can cause additional complications. Therefore, the demand for regeneration of joint cartilage has led to an alternative approach called "orthobiologics". This alternative is based on cellular and molecular components capable of inducing and promoting tissue repair. Products derived from adipose tissue have been studied as an excellent source of orthobiologics in an attempt to promote joint cartilage repair. However,

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Manuscript source: Unsolicited manuscript

Specialty type: Orthopedics

Country/Territory of origin: Brazil

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E

Received: October 14, 2020
Peer-review started: October 14, 2020
First decision: December 24, 2020
Revised: January 11, 2021
Accepted: February 28, 2021
Article in press: February 28, 2021
Published online: February 28, 2021

P-Reviewer: Grawish ME, Mehanna RA
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH

the lack of standardization regarding collection and processing protocols presents a challenge for the generalization of study results and determination of effectiveness. To the best of our knowledge, orthobiologics derived from fat have not yet been classified. Therefore, this manuscript proposes the HGS classification system which aims to describe certain parameters that are relevant to the quality of organic products regarding harvesting techniques (H), graft type (G), and number of centrifugations (S). The more parameters used would imply greater characterization and complexity of the evaluation of the biological product used. The HGS classification may provide a valuable contribution to the understanding of clinical procedures and research results, aiming to ultimately usher in a standardization of optimal practice.

Key Words: Orthobiologics; Adipose tissue; Mesenchymal stem cells; Regenerative medicine; Musculoskeletal disorders; Orthopedics

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Core Tip: Chronic musculoskeletal disorders remain one of the top causes of pain and disability in the adult population. The demand for musculoskeletal tissue regeneration has led to an alternative approach referred to as orthobiologics, which is based on cellular and molecular components capable of inducing and enhancing regenerative cascades in injured tissues. Adipose tissue-derived biological components are current orthobiologic tools being extensively studied and evaluated by regenerative medicine experts for their potential to treat musculoskeletal conditions. Since adipose tissue-derived orthobiologics have not yet been classified, to the best of our knowledge, this manuscript aims to propose the HGS classification system.

Citation: Macedo RDR, Fonseca LFD, Lana JFSD, Mosaner T, Purita J, de Andrade M, Rodrigues LM, Centurion P. Biofat grafts as an orthobiologic tool in osteoarthritis: An update and classification proposal. *World J Meta-Anal* 2021; 9(1): 29-39
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/29.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.29

INTRODUCTION

Osteoarthritis (OA) is a common and very debilitating pathology, especially in elderly individuals[1], but it can also affect younger individuals due to numerous factors[2]. Currently, with the aging of the population and with the increase in physical activities, skeletal muscle problems have also been increasing in equal proportion[3].

OA is more prevalent in weight-bearing joints with the highest incidence in the knees; it may be idiopathic or secondary. The initial treatment consists of physical rest, modification of physical activity, weight loss, physiotherapy, orthosis, anti-inflammatories, analgesics[4] and, historically, injections of corticosteroids to relieve symptoms[3]. However, these treatments are palliative by managing only the symptoms. Currently, the use of hyaluronic acid (HA) can also contribute to decrease in pain and improvement of mobility due to its viscosupplementation potential. Additionally, high molecular weight HA is administered to further reduce oxidative stress and cell apoptosis[5,6]. HA is also thought to be a signaling molecule. These biological roles range from a purely structural function in the extracellular matrix to regulation through effects of cellular behavior *via* control of the tissue macro- and microenvironments, as well as through direct receptor mediated effects on gene expression.

In the field of orthopedics, when conservative treatment fails, the surgical procedure is usually indicated. In most cases arthroplasty may be recommended, however, it is known that these interventions have numerous complications and comorbidities that can lead to death in up to 5.6% of individuals operated[6].

In view of the fact with the failure in conservative treatment and the aggressiveness of surgical intervention, the use of orthobiologics has become an outlet to avoid or postpone surgeries[7,8], relying on the anti-inflammatory and reparative properties of

the patients' own cells for treatment of the injured tissues[9,10].

Envisioning a new direction in cartilage regeneration, innovative therapies are being introduced, including platelet-rich plasma (PRP), bone marrow aspirate concentrate (BMAC) and adipose tissue with its different products[5,11].

Mesenchymal cells can be found in many tissues[12], but they are commonly extracted from bone marrow and adipose tissue. These cells are being studied extensively because they offer an alternative in the treatment of orthopedic pathologies since they have the ability to differentiate into other cell types[13]. They have great capacity for plasticity and self-renewal, in addition to exerting anti-inflammatory and immuno-modulatory roles[13]. However, the paracrine effects elicited by growth factors, cytokines, bioactive lipids as well as exosomes and microvesicles (which have been found to promote regeneration of damaged tissue) all play a fundamental role in orthobiological therapies[14,15].

The choice of the extraction site may have a limiting character in the success of regenerative therapy. Among the donor sources, adipose tissue sources have a prominent place in the literature. Since 2001 Zuk *et al*[16,17] have shown that the stromal vascular fraction (SVF) contains stem cells capable of differentiating into cartilage, bone, muscle and fat. It has been shown that the immunophenotypes of BMAC and SVF cells are more than 90% identical[18], however, one of the advantages of using fat is that it is easy to collect and it also offers more alternative extraction sites in the body. Additionally, if one compares 100 mL of BMAC with 100 g of adipose tissue, one may find 300 times more mesenchymal stem cells (MSCs) in adipose tissue[18,19]. Currently aiming at the paracrine understanding of mesenchymal cells, secretomes have been studied with analysis of pro-inflammatory cytokines such as interleukin 1B (IL-1B) which has a very important role in the pathogenesis of OA. IL-1B dramatically increases expression of matrix metalloproteinases that contribute to the degradation of articular cartilage; chondrocytes stimulated by IL-1B increase the secretion of nitric oxide and prostaglandins E2, which are major inflammatory mediators[20]. Mesenchymal cells display anti-inflammatory effects mainly by paracrine signaling action[21].

In the present study, we will enumerate and describe the possible products of adipose tissue and their application in the treatment of OA as a regenerative therapy as well as the manipulation processes involved in the acquisition of mesenchymal cells.

Orthobiologics have been discussed in the literature with promising results, however, the lack of standardization regarding the methods of obtaining and processing the cells and associated components have led to uncertain conclusions in terms of efficacy and ability to generalize outcomes[22]. More specifically, the main components of orthobiologics, such as PRP, for example (platelet concentrations, growth factors, and cytokines), may vary based on the processing method, which might affect anabolic and anti-inflammatory properties and, consequently, lead to inconsistent outcomes[23]. Thus, the need for standardization and classification of orthobiologics is imperative for understanding procedures and dissemination of research outcomes. A classification system has been developed for PRP[22] and for BMAC[24]. However, no such classification exists for adipose tissue as an alternative orthobiologic tool. Thus, the purpose of this paper is to present a proposal for a classification system for adipose tissue-derived orthobiologics.

MESENCHYMAL STEM CELLS

MSCs exhibit the potential ability to differentiate into mesodermal cell lineages (*e.g.*, cartilage, bone, fat, muscle, meniscus and tendon)[25], which is fundamental for the regeneration process. Moreover, these cells elicit paracrine effects, and are therefore capable of altering their local microenvironment[26].

Given the varying MSC markers that laboratories may use to characterize these cells, there is a lack in standard phenotypic criteria. This heterogeneity is also due to the fact that MSCs are able to express a range of cell-lineage-specific surface antigens that may differ depending on the culture preparation, duration, or plating density[27]. However, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have proposed minimal criteria to characterize MSCs, which comprise the following attributes: Cells must be plastic-adherent when maintained in culture; Must be able to differentiate *in vitro* into chondroblasts, adipocytes and osteoblasts; and Must express CD105, CD73 and CD90 whilst lacking expression of CD45, CD11b, CD34 or CD14, CD79α or CD19 and HLA-DR surface

molecules[28].

MSCs lack significant immunogenicity and can be easily isolated, which allows allogenic transplantation, but the MSC-exosomes do not appear to trigger immune-rejection and could be the optimal treatment in specific clinical scenarios in the future[29].

Under allogenic circumstances, these cells should be considered immune evasive. However, the effects of MSCs in cellular-based therapies depend on the ability of these cells to home and engraft (long-term) into the target tissue[30].

Cells from injured tissue release chemokines responsible for MSC recruitment. Once in the target tissue, MSCs are able to modulate wound-healing responses by reducing apoptosis and fibrosis, attenuating the inflammatory process. They also stimulate angiogenesis, cell proliferation and differentiation *via* paracrine and autocrine pathways[31]. These properties are attributed to the ability of MSCs to release key agents, such as vasculoendothelial growth factor, transforming growth factor beta, stromal-derived factor 1, and stem cell factor, among others. Also, they induce a downregulation of pro-inflammatory cytokines, including IL-1, IL-6, interferon-γ, and tumor necrosis factor alpha (TNF-α)[32,33].

MSCs also possess immunomodulatory properties as they are able to inhibit the activation of type 1 macrophages, natural killer cells, and both B and T lymphocytes[34].

BIO FAT GRAFT

It is currently known that adipose tissue is one of the best sources of MSCs, which may also be referred to as MSCs or adipose-derived stem cells (ADSC)[35]. The benefit of using adipose tissue-derived products in OA, as previously mentioned, is its paracrine action with secretion and activation of hormones such as leptin, resistin and cytokines such as TNF alpha in mediating the chronic inflammatory process. Another advantage of using adipose tissue as a source of mesenchymal cells is the fact that there is no decrease in the number of these cells with age[36].

In order to obtain all the benefits offered by ADSCs, there are some ways to prepare adipose tissue-derived products. The techniques involved in this procedure will be reviewed immediately in the following sections of this manuscript.

One noteworthy observation is that, unfortunately, there is a lack of standardization demonstrating which types of methods are most effective for obtaining and isolating mesenchymal cells from fat. The harvest result may sometimes be referred to as "Stem Cells-poor" aspirated fat.

OBTAINING FAT

In order to obtain adipose tissue, one may find convenient extraction sites described in the literature, such as the abdomen, thigh, hip, buttocks, and infra-patellar fat pad. The lower abdomen and hip regions have higher cell quality when compared to other locations; however, cell viability does not change with the donor site[19].

There are four key steps that must be followed in order to obtain MSCs from adipose tissue, and the careful handling in order to optimize the harvesting procedure. Step 1: Donor area, how it is treated, technology utilized; step 2: Aspiration, closed or non-closed system; step 3: Processing, SVF isolation; and step 4: Injecting.

Sub cutaneous fat tissue for regenerative purposes may be obtained through the following procedures.

Step 1: Donor area preparation

Fragment resection[37,38]: In this procedure, the removal of a block of fat from any donor area occurs; The advantage is the preservation of all the components including extracellular matrix and regenerative elements. With this fragment, the whole process of enzymatic digestion is prepared for separation of the mesenchymal cells and posterior cell culture. The major disadvantage is that enzymatic digestion affects the number of MSCs during manipulation. Elaborating, cell washing after collagenase administration may also imply the use of fetal bovine serum, which can dilute the concentration of MSC. Collagenase does not preserve the extracellular matrix where all the necessary elements or regenerative factors are found in the exact proportion and concentration. The incision made at the extraction site for acquisition of the necessary fat volume utilizes a closed system (air exposure), which can increase apoptosis.

Suction-assisted lipectomy (SAL): This technique is combined with tumescent infiltration with saline solution + anesthetic + adrenaline, in the subcutaneous area of the donor site in order to form a swelling and thus facilitate the liposuction process in addition to reducing bleeding[39].

The advantage is that this is a closed system, with less incision at the donor site. The disadvantage is that the tumescent infiltration dilutes the proportion and concentration of the regenerative cells and particles, requiring the need for additional harvest process maneuvers. The mechanical disruption mechanism is also quite destructive towards the adipose tissue[29].

Liposuction with ultrasound[19,40]: Adipose Tissue sonication process allows obtainment of only microclusters of cells and no breakdown of adipose tissue[41]. The major disadvantage is that this is not a closed system.

Laser liposuction: The use of the specific wavelength (1210-nm) laser to dissolve the bound cells (adipocytes, stem cells, extracellular matrix) to the connective tissue before aspiration facilitates suction and may promote cellular photostimulation, *via* a photochemical property of the infrared light 1210-nm. Centurión *et al*[29] demonstrate that the SAL technique can cause cell lysis; the use of certain lasers with affinity for water can cause tissue damage by increasing the permeability of the adipocyte cytoplasmic membrane[42].

With the use of a laser with a wavelength of 1210 nm in the adipose tissue, a selective photostimulation effect has been described. This technique may guarantee up to 98% viability of adipocytes, remaining unchanged. Additionally, since it relies on photobiomodulatory effects, it is also proven to generate an increase in mitochondrial activity[29,42].

In all of the techniques there is minimal patient discomfort and low morbidity in the extraction site[43]. Oedayrajsingh-Varma *et al*[19] demonstrated that the use of ultrasound requires a greater amount of time for the growth of cells in culture when compared to fat fragmentation and SAL.

Step 2: Aspiration: closed or non-closed system

Closed system uses a syringe connected to the cannula, avoiding air exposure.

Non-closed system: Using a syringe connected to the cannula but exposes the material to air during processing maneuvers. An aspiration machine may also be utilized however air exposure is increased during the collection process and, therefore, the apoptotic response of cells.

Step 3: Processing adipose tissue and SVF isolation

Minimal-grade manipulation (MGM) or high-grade manipulation (HGM) (C).

MGM: This refers to techniques that performed during the same surgical session and inside the same operating room, but only if the tissue is harvested, treated, and re-implanted.

Among the techniques listed as MGM, one can find 3 ways to treat Fat: (1) Mechanical disruption: Decantation, filtration, centrifugation, emulsification, and other techniques described as Coleman's technique. These include direct ultrasonic cavitation, cell assisted lipotransfer, Nanofat (Tonnard), SAL; (2) Exogenous enzymatic digestion: Exogenous collagenase is used, increasing the cost, the manipulation and the risk of side effects if any enzymatic residue is present in the final product. Devices like Cytori Celution and Unistation may be utilized, as examples; and (3) Endogenous Enzymatic collagenase activation - One STEP™ technique: This involves infrared 1210-nm laser activation of the endogenous quiescent collagenase, present in the subcutaneous tissue. Activation is made in a single step. This process denatures the connective tissue, liberating the MSCs and preserved adipocytes, with mitochondrial stimulation *via* cytochrome C oxidase. After adipose tissue aspiration, a simple centrifugation is performed in order to isolate the SVF, in a closed system with minimal manipulation.

HGM: This includes characterization, expansion, cultivation and all the other complementary laboratory techniques. In some countries HGM is strictly prohibited.

METHODS OF ISOLATION OF MESENCHYMAL CELLS

SVF isolation by enzymatic digestion

The use of fat digestion through collagenase is the most widely used method when the objective is to culture and expand mesenchymal cell[44] Collagenase separates fat into 2 distinct layers: The floating fraction of mature adipocytes, and the cellular components in the lower aqueous portion[45], which are separated by centrifugation, gravity or *via* the use of filters. However, the presence of collagenase in injectable products for the treatment of orthopedic conditions is not allowed by the Food and Drug Administration, so it is well-suited for cell culture and expansion studies only. There are also additional methods involving the administration of lecithin to obtain SVF.

SVF isolation without enzymes

Due to the regulatory issue related to the isolation of mesenchymal cells with collagenase, other methods were considered in order to try to achieve this isolation with an acceptable level of effectiveness.

Most of these techniques involve a mechanical breakdown of the adipose tissue to obtain SVF through microfragmentations, however the cell yield is much lower when compared to the enzymatic method because adipocytes establish strong bonds with collagen that are not easily released with mechanical force[46].

Authors have described the method of filtering and emulsifying fat through small filters called "nano-filters"[47], which is why the term "nano-fat" is used. This method consists of the passage of fat through some filters, which causes emulsification of this product. Since some researchers have previously identified the presence of mesenchymal cell markers, these alternative biological products are currently being widely used in aesthetic medicine with promising results[48].

Perhaps the combination of techniques with centrifugation and filtration can produce a higher concentration of mesenchymal cells, eliminating the use of enzymes. This reduces the cost, processing time and remains in compliance with the regulatory bodies for therapeutic use. Currently, there are some kits on the market that assist in cell separation, using filtration with or without saline wash, whilst other kits employ centrifugation steps. However, there is still a lack of standardization and a better comprehension of the product obtained from these different devices.

Automated devices for isolation of SVF

Automated devices are already available on the market for the separation of mesenchymal cells from liposuction. The first system described was in the USA, "Cytoris Celution"[49]. Lastly there is a non-enzymatic method for the acquisition of SVF referred to as ultrasonic cavitation.

PROPOSAL OF A NEW CLASSIFICATION SYSTEM FOR ADIPOSE TISSUE-DERIVED PRODUCTS: THE HGS CLASSIFICATION

The lack of standardization of adipose tissue-derived products for regenerative medicine has emerged, thus the need to classify the processing methods in regards to quality and procedural details has been established[23]. Classification of such factors would allow for procedural standardization and interpretation of both clinical results and research findings. The HGS classification system comprises the three main techniques involving liposuction for harvesting, which is represented by the letter H; Graft, which is represented by the letter G (SVF isolation method); and the letter S which refers to spin, indicating the inclusion or exclusion of centrifugation.

The HGS classification is focused on whether the cellular and molecular content present in the product is evaluated and described, increasing the complexity of description/characterization. For each letter there is a sub grouping, as follows.

For letter H (Harvesting –liposuction): (1) SAL; (2) Ultrasound assisted; and (3) Laser assisted.

Letter G (represents the method for isolation of the SVF- Graft) there is (1) Decantation (washing); (2) Microfragmented (Nanofat); (3) SVF non-enzymatic; (4) SVF enzymatic; and (5) Automated devices.

In this description (H), we have yet another subgroup which is also presented in the form of numbers for cell characterization, described as follows: (1) Product is to be collected and injected with no additional analysis; (2) The cellular content would be assessed by a cell counter, which would enable quantification of mono- and

polymorphonuclear cells, giving the number of total nucleated cells; (3) Dosage of molecular content, such as interleukins and/or growth factors is made by multiplex platform or ELISA technique; (4) Indirect quantification of MSC number measured through colony forming unit (CFU) in culture; (5) Phenotyping of MSC and HSC for characterization through flow cytometry – it is wise to use a full panel for the CD (cluster of differentiation) surface markers, especially of the MSC since there many markers for positive and negative evaluation; and (6) For the complete characterization of MSC, the *in vitro* differentiation into the mesodermal trilineage is necessary, which means the induction of chondrocytes, adipocytes and osteocytes.

For letter S (Spin- inclusion or exclusion of centrifugation steps): (1) Yes; and (2) No.

The representation of the HGS classification is shown in Table 1.

The purpose of classification is to be descriptive for collection, graft type, characterization and centrifugation. This should be considered in order to standardize the publications and facilitate the understanding of the type of fat products, especially in terms of quality.

The idea of this classification is that for each type of fat products used the increase in the number indicates higher complexity of the evaluation of the biological product obtained. For example, when a study or procedure with adipose tissue reports that the adipose tissue was collected *via* SAL, with decantation and injected after centrifugation, it would be classified as H1, G1-1, S1, according to the HGS classification. On the other hand, if it is collected with the aid of ultrasound, microfragmentation without centrifugation and cell count is performed, then it would be classified as C2 H2-2 S2. This is applied successively, always in correspondence with the subgroups of the letter H (3 subgroups), the two subgroups of the letter G being the first related to the separation technique, and the second subgroup related to the characterization of adipose tissue, and finally the two subgroups of the letter S. It is worth remembering that for letter G in the second subgroup, when we characterize the sample it is possible to have more than one number. For example, the product went through cell count and CFU, so in this case we would use the numbers 2 and 4 in the second subgroup. An example is presented below.

Liposuction procedure using laser (H-3); enzymatic digestion technique for separation of mesenchymal cells (G-4) with cell count (2), dosage of cytokines (3), CFU (4) and MSC phenotyping (5); and lastly, centrifugation (S). Result: H3, G4 (2-5), S1.

DISCUSSION

Aiming to improve the quality of life in patients with musculoskeletal disorders, regenerative medicine has been occupying a space with novel research for the improvement of pain and tissue repair[50]. Mesenchymal cells obtained from adipose tissue exert important roles and have recently been gaining prominence in numerous publications. In 2006, Nguyen *et al*[51] revealed improvements in quality of life questionnaires from patients with knee OA (KOA) who were treated with SVF combined with PRP. In a randomized double blind clinical trial, Hong *et al*[52] showed the efficacy of SVF (with collagenase) compared to hyaluronic acid in patients with KOA. In 2017, Yokota *et al*[53] revealed that the scores used to analyze the improvement parameters were significantly higher 1 mo after the infiltration of SVF in the knees of osteoarthritic patients. In the literature, we also found a review including 17 articles evaluating SVF for OA, with the majority of fat products coming from the abdominal region and being processed with the use of collagenase. Most of the results were satisfactory but the articles compiled in this review do not have a level of evidence capable of validating the efficacy of the treatments.

Based on some current articles, the use of mesenchymal fat cells appears to be extremely safe with encouraging results when functional outcomes and pain alleviation are taken into consideration, suggesting that this biological approach may play a promising role as a suitable treatment for OA.

It is known that mesenchymal cells elicit a broad range of effects favoring intra-articular modulation and delaying degeneration through their paracrine action as well as offering the possibility of tissue repair due to the presence of stem cells in the vascular stroma. Although most studies demonstrate superior results with mesenchymal cell culture, Jurgens and Col obtained excellent results with the use of SVF in a single procedure[54-56], however further studies comparing the two modalities must be performed in order to propose a definitive answer to this question.

As is the case with the majority of biological products, we verified a wide variety of preparation techniques, collection sites, non-standardized therapeutic protocols and

Table 1 HGS classification system

HGS classification system		
H	**H**arvesting (Liposuction)	(1) Suction-assisted lipectomy; (2) Ultrasound assisted; and (3) Laser
G	**G**raft: (1) Decantation (washed); (2) Microfragmented (Nano); (3) SVF non enzymatic; (4) SVF enzymatic; and (5) Automated devices	(1) Collection and injection; (2) Cell count; (3) Dosage of cytokines (GF and/or IL); (4) CFU; (5) MSC phenotyping; and (6) Differentiation evaluation
S	**S**pin	(1) Yes; and (2) No

SVF: Stromal vascular fraction; MSC: Mesenchymal stem cell; CFU: Colony forming unit.

concomitant treatments, which compromise the comparison between studies[54]. Due to the numerous forms of presentation and approach, there is a lack of standardization in order to render the results more effective. To affirm which technique is better for MSC harvesting, it is necessary to analyze and compare MSC both in quantity and quality (% of CD markers). Another important factor is the presence/preservation of extra cellular matrix, paracrine factors, exosomes, and cytokines in the final product. Therefore, the use of expanded cells for the scope of science may bring greater security because it allows greater control of the product with cell count and the possibility of better characterization of the sample[57,58].

New technologies appear to enhance harvesting, like the laser technology, but current devices with specific wavelengths and absorption/affinity for water may not be adequate due to possible lipolytic reactions by increasing the temperature (photo-thermal effect). Only a novel infrared laser 1210-nm wavelength (One STEP™ technique) offers an absorption/affinity for fat *via* photochemical properties[42].

The quality of the lipoaspirate is not affected when using different cannula size or negative pressure in the SAL procedures[59]. What may affect the quality and quantity of the aspirated material is the mechanical disruption mechanism of the technique.

CONCLUSION

Given all the information, physically debilitating conditions such as OA are still highly challenging pathologies for professionals seeking a definitive treatment. The search for ideal biologic products has become an increasingly common practice but this goal has not been reached just yet, despite the growing number of clinical trials envisioning an effective treatment protocol. In scientific terms, however, it is safe to infer that the levels of evidence for the use of SVF in the treatment of OA are still under early development but certainly promise great results in the near future.

Although studies using adipose tissue for the treatment of various musculoskeletal disorders have shown promising clinical results, inconsistent preparation methods with deficient reporting has led to questionable outcomes with respect to generalization and reproducibility. In order to optimize the efficacy and safety of fat products, and to allow validation and standardization of such products, studies should report stepwise descriptions of the preparation protocol and additional information to further classify the product used. The HGS classification focuses on describing parameters that are relevant for the quality of biological products, such as the collection technique, cell count and molecular content dose. The HGS classification would contribute to a greater understanding of the type of harvest, clinical procedures and research outcomes and, over time, lead to a standardization of best practice. Together, we believe that the HGS classification proposal is an easily recalled and useful method for the classification of fat products.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 40-44

DOI: 10.13105/wjma.v9.i1.40

ISSN 2308-3840 (online)

MINIREVIEWS

Non-invasive diagnosis of Crohn's disease: All that glitters is not gold

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Author contributions: Khorshid M contributed to the idea and the primary draft of this study; Elkady MAK contributed to the collection and analysis of reference articles; Abdelkarim R wrote the manuscript; El-Nady M revised the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Manuscript source: Invited

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Abstract

Crohn's disease (CD) is associated with occurrence of inflammation in the digestive tract. Diagnosing intestinal bowel diseases can be difficult because bowel disease can be tricky as it does not have unique symptoms. Endoscopy and histopathological tests play a crucial role in the diagnosis and management of inflammatory bowel diseases. Various techniques can be used to diagnose CD. Nevertheless, the diagnosis of CD mostly requires having patients in the hospital. During the SARS-CoV-2 pandemic, that might not be very feasible, as minimizing contact is essential, but can an alternative diagnosis technique be enough to provide a definitive diagnosis?

Key Words: Crohn's disease; Inflammatory bowel disease; Diagnosis; Noninvasive; Histopathology; Overdiagnosis

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Core Tip: Diagnosing intestinal bowel diseases can be difficult because bowel disease can be tricky as it does not have unique symptoms. Endoscopy and histopathological tests play a crucial role in the diagnosis and management of inflammatory bowel diseases. However, various techniques can be used to diagnose Crohn's disease.

Citation: Khorshid M, Elkady MAK, Abdelkarim R, El-Nady M. Non-invasive diagnosis of

manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Egypt

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 17, 2020
Peer-review started: December 17, 2020
First decision: January 10, 2021
Revised: February 3, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Fiocchi C
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li JH



Crohn's disease: All that glitters is not gold. *World J Meta-Anal* 2021; 9(1): 40-44
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/40.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.40

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory condition that affects any part of the gastrointestinal tract, and is characterized by the development of fistulas or strictures[1]. The incidence of CD varies around 5/100000, a figure that is associated with residence in urban areas[2]. CD has nonspecific symptoms and commonly shared symptoms with other intestinal bowel diseases, such as ulcerative colitis. This makes the diagnosis of CD challenging with many patients, especially those with mild and moderately severe disease. The diagnosis of CD is based on various clinical, histological, and laboratory findings. Histopathology can be used as a confirmatory method for diagnosing CD along with imaging modalities. Additionally, diagnostic modalities are used either for disease severity evaluation or for monitoring the response to treatment[3]. However, histopathology is not always accessible, and even reaching the site of inflammation for taking biopsies is not always feasible. Differentiation from other causes of small intestine inflammation (*e.g.*, lymphomas, Behçet disease, and tuberculosis) is crucial as immunosuppression induced by CD therapies may aggravate those conditions and result in additional, unnecessary risk of serious adverse events. This highlights the necessity of having various procedures to confirm the diagnosis of CD. Recently, numerous useful tools and techniques have emerged. However nowadays, under this austere environment, an accurate technique with minimal patient contact is extremely important, but high accuracy with a definitive diagnosis also remains vital.

HISTOPATHOLOGICAL TESTING

The presence of non-caseating granuloma could confirm the diagnosis in the setting of a typical clinical presentation. Biopsies may be taken during upper endoscopy (including a portion of the jejunum), colonoscopy (including the terminal part of the ileum), and during surgical exploration in complicated cases. However, inflammation may not be accessible by upper endoscopy or colonoscopy, especially if surgical exploration is not indicated. This hinders taking a biopsy, makes it less convenient, and requires using an alternative modality.

DEVICE-ASSISTED ENTEROSCOPY

The use of device-assisted enteroscopy (DAE) is mandatory when the biopsy location is unapproachable[4]. DAE has an outstanding advantage of allowing deeper intubation of the small intestine. However, it is of limited availability due to a prolonged examination time and carries an increased risk of perforation, especially in the situation of markedly inflamed ulcerated mucosa. Most DAEs are performed in tertiary hospitals, and limited availability results in frequent long queues that delay the diagnosis of such cases.

VIDEO CAPSULE ENDOSCOPY

Video capsule endoscopy (VCE) is the least invasive technique compared with the others. It is now taking on a more prominent role in the detection of small intestinal lesions and monitoring disease progression. With its increased availability, straightforward interpretation, high-quality imaging, simplicity, and most importantly, noninvasiveness of the procedure, VCE has become a highly recommended modality in the European Crohn's and Colitis Organization guidelines[5]. Nevertheless, the inability of taking biopsies, relatively high cost, and the fear of retention when in doubt of strictures have limited its use. Esaki *et al*[6] conducted a nationwide case-control study that illustrated the presence of distinctive

signs for CD that may ease the diagnosis without the need to biopsy. The signs include cobblestoning (a cobblestone appearance), many longitudinal ulcers and erosions, and circumferential and longitudinal diminutive lesions[7].

MAGNETIC RESONANCE ENTEROGRAPHY

Magnetic resonance enterography (MRE) recently became a commonly used diagnostic tool for CD following development of explicit criteria of evaluation and the emergence of the magnetic resonance index of activity (MaRIA). MaRIA is an improved scoring system that provides a more accurate evaluation of the disease insult to the intestines and the detection of complications, making MRE an essential tool for assessing the patient's condition[8]. Although MRE avoids the operator-dependent bias associated with abdominal ultrasound of the bowel, it has a few drawbacks. The process of the procedure may not be tolerated by the patient and the availability of the machine and the experience of the operator and interpreter may be limited[9,10].

LABORATORY TESTS

CD can be suspected by classical clinical symptoms and signs, involving either the gut or extraintestinal manifestations. From the time of the presentation of the patient and throughout the patient journey, a few laboratory tests aid the process of evaluation. During the diagnosis phase and on follow-up, C-reactive protein, calprotectin, complete blood cell counts, and ferritin, are regularly requested and are proven to correlate with disease activity[11]. Vitamin D deficiency, positive anti-*Saccharomyces cerevisiae* antibodies, low albumin, and the presence of red blood cells and pus in the stool analysis may point to the state of gastrointestinal inflammation. All these tests can provide a better insight to the physicians. In addition to the chance of raising doubts of the CD diagnosis, the tests can also be used to evaluate disease activity and monitor the response to treatment[12,13].

Apart from the guidelines and the current literature recommendations, to gain extra time until the CD diagnosis is proven and when the treating physician is in doubt, many physicians tend to start a trial treatment and judge the response. This trial treatment includes initiation of glucocorticoids in lower than recommended doses for treatment of active CD. However, although we may encounter this in our regular practice, to our knowledge, its benefit was never proven in a scientific publication. The concept of a steroid trial was borrowed from rheumatologist and dermatologist treatment of suspected immune disorders, and when patients with inflammatory bowel disease present with extraintestinal manifestation.

DIAGNOSIS DURING CORONAVIRUS DISEASE 2019

During the early months of the coronavirus disease 2019 (COVID-19) pandemic, healthcare services for nonemergency patients were less accessible. Endoscopy was reserved for those who had alarming signs only. We recently noticed an increase of patients referred with a diagnosis of CD without histopathological evidence. Their evaluation had been completed by either MRE or by other endoscopic modalities, and many of those patients were falsely misdiagnosed[14]. Hence the need of a confirmatory technique is vital, and the specificity and sensitivity of each available technique are compared in Table 1. The comparison shows that VCE is the leading diagnostic modality and has the potential to meet current needs[15].

CONCLUSION

To conclude, the healthcare system has been under severe stress from the current pandemic. COVID-19 has resulted in restrictions on asking patients to go to the hospital and to limit and avoid prolonging the time spent there. Patients need to agree to very precise procedures and only for crucial emergencies. CD is not characterized by specific symptoms and can be mistaken for other bowel disorders because of shared

Table 1 Accuracy of currently used diagnostic modalities

Tool	Ref.	*n*	Sensitivity	Specificity
VCE	Monteiro *et al*[7]	36	90%	100%
MRE (MaRIA)	Roseira *et al*[9]	84	90%	98%
CRP	Mosli *et al*[13]	2499	49%	92%
Calprotectin	Rokkas *et al*[12]	2822	82.4%	72.1%

The modalities used in each study and the number of cases that were recruited in each are shown. Sensitivity and specificity were calculated on that basis. The study by Rokkas *et al*[12] using calprotectin had the largest number of patients but reported the lowest specificity. On the other hand, Monteiro *et al*[7] who used video capsule endoscopy had the highest sensitivity and specificity. CRP: C-reactive protein; MaRIA: Magnetic resonance index of activity; MRE: Magnetic resonance enterography; VCE: Video capsule endoscopy.

symptoms. Various well-established imaging modalities used in diagnosing CD. However, as they can be a bit risky, using safe, combined techniques could be our go-to at this time. Having a confirmed diagnosis is of ultimate importance to provide the right treatment. The diagnosis of CD is sometimes not accessible because of the need for histopathologic confirmation before initiation of treatment. Histopathology has limitations both in occasional difficulty in taking a biopsy and in a tentative diagnosis. In the aspect of the guarantee of the high diagnostic value of other modalities, such as MRE and VCE, combining both of them and supporting them with laboratory tests may decrease the need for histopathology in diagnosing CD. Future research may fulfil this strong demand by developing a model for noninvasive diagnoses of CD. The potential of having a noninvasive technique could not only be promising for the time being, but also more comfortable and less stressful for patients compared with regular biopsies and other invasive techniques.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 45-50

DOI: 10.13105/wjma.v9.i1.45

ISSN 2308-3840 (online)

MINIREVIEWS

Magic and forensic psychiatry: A case study and review of the literature

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Author contributions: Vyshka G and Simoni S collected data, drafted the manuscript, reviewed the literature and wrote the final version; both authors approved the initial and the revised manuscript in its present form.

Conflict-of-interest statement: Nothing to declare.

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Manuscript source: Invited manuscript

Specialty type: Psychiatry

Country/Territory of origin: Albania

Peer-review report's scientific

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Abstract

Forensic psychiatrists routinely appear in courts for crimes that have been perpetrated by mentally ill patients or when the defendant is suffering from a major psychiatric disorder. However, committing crimes under the presumed effects of magic and witchcraft is uncommon. A variety of delusional thoughts and personality disorders have been imputed in aggressive behaviour that might culminate in homicide. The patterns of a premeditated homicide, as well as the aberrations of the interpersonal relations, as a rule require long-term and close contact. As such, the family is a setting where such occurrences have been reported. We describe a case of first-degree murder by a woman who killed her mother-in-law after accusing her repeatedly of sorcery and witchcraft. Such complicated cases cannot be resolved without the help of forensic experts, whose role remains multifaceted and of particular importance.

Key Words: Belief in magic; Witchcraft; Criminology; Forensic psychiatry

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Core Tip: Belief in magic and spelling objects (witchcraft) are rarely object of the prosecutors' investigations, a fact that may be related to the public abhorrence of the phenomenon itself. Family is a highly vulnerable setting, when there are no compensatory mechanisms for altercations and everyday quarrels. Such complicated cases cannot be resolved without the help of forensic psychiatrists, whose role remains multifaceted and of particular importance.

Citation: Vyshka G, Simoni S. Magic and forensic psychiatry: A case study and review of the

quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: December 26, 2020
Peer-review started: December 26, 2020
First decision: January 18, 2021
Revised: January 24, 2021
Accepted: February 22, 2021
Article in press: February 22, 2021
Published online: February 28, 2021

P-Reviewer: Niveau G
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH



literature. *World J Meta-Anal* 2021; 9(1): 45-50
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/45.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.45

INTRODUCTION

Belief in magic refers to the persuasive omnipotence of a thought. In other words, if you simply think about something, it may turn into reality. Magic and belief in it, as a normal part of earliest human communities, were once considered as harmless, and the rituals were attributed to well-educated people of a certain reputation. Cultural influences, evolution, societal progression, and age-related changes are some, but not the only factors influencing the popularity of magical thinking[1,2]. Once considered as a sophisticated way of thinking, the belief that spirits and demons may live within us and cause mental health issues is now abolished and regarded as a mental disorder. In general, societies shifted from sanctifying these practices in the antiquity toward condemning them in the medieval period; even witch-hunt has expanded to local areas, such as Salem in Massachusetts among many examples[3].

Forensic medicine professionals' opinion, particularly forensic psychiatrists, are routinely summoned in courts when perpetrators act under delusional influences. However, magic does not straightforwardly suggest the existence of a delusional disorder, as a plethora of factors contribute to this distorted way of thinking: Religious delusions can be a very good example[4,5]. Moreover, cultural background plays a very important role in the overall understanding and explanation of magical practices. Magic was once practiced in well-accepted rituals. Even today, some societies are familiar with these practices or at least have a less rigid view about them due to their harmlessness[6,7].

Generally, there are various criminological views about this phenomenon. If magic is a distorted way of belief and thinking, it may be explained under anthropological terms and remain an eternal curiosity to the mankind. Renowned anthropologists, including Frazer, Durkheim, and many others, have made substantial contributions in this field[8,9]. One should pay attention to the medically important fact that if magic and magical thinking still trigger ethnological and anthropological curiosity, they may become a psychiatric concern in Western societies. The Diagnostic and Statistical Manual of Mental Disorders (DSM) considers magical thinking as the delusional part of an odd belief, which can be classified as a schizophrenic symptom or at least a schizotypal archetype[10].

The killing of a family member is not an infrequent event. However, in the majority of cases, the perpetrators are charged and sentenced for crimes of futile reasons or impulsively committed. Families under stress are clearly at a higher risk of violence, and frustration can lead to a spiral of interpersonal aggression and violent acts, culminating into a murder[11]. By studying the traits of murder within a family, one might wonder how easily an everyday situation can degenerate into altercations and use of force. The so-called "ordinary murderer" might be provocative by a simple act that will provoke the tragedy[12]. Nevertheless, acting under the influence of magic or spells is an exceptional event. Such circumstantial combinations rarely lead to a serious crime, such as killing within the family.

CASE STUDY

The Albanian public opinion was struck some years before from a crime within family, with a mother-in-law ruthlessly strangulated and poisoned with Phostoxin by her son's wife. The case received wide coverage from the media and an important bulk of the crime details (if not its entirety) was published and republished in all local journals and other mass media.

After long and repeated skirmishes and quarrels, the wife of the victim's son decided to come to an end. The perpetrator was a pregnant, newlywed, 26-year-old rural female, who was poorly educated, and easily gullible. With a previous miscarriage and a highly expected progeny, she was convinced that her mother-in-law was responsible for all her unhappiness, including her previous miscarriage, which was extremely unfortunate for herself and her husband. Belief in magic and alleged witchcraft were highly probable causes of crime. After being arrested and indicted

from the state attorney, the perpetrator made the following statements: "…While we were adjusting the water supply in the terrace some months earlier, we found a bottle with a big piece of meat plunged inside…My husband and I immediately doubted that someone was doing witchcraft or some kind of magic spell to make us suffer. We had heard about people doing such things. After that day, we continuously discovered strange objects in our bedroom, including animal teeth over the nightstand, and our suspicion became stronger. Also, I found hanging socks of mine and of my husband at the window with human hair inside…Our marital relationship was getting worse day by day…".

This crime was perpetrated in a rural family with a low-to-middle economic status. The murder was premeditated long before, as the perpetrator had purchased a strong poison (Phostoxin) several days before the crime. She prepared a lemonade drink with the poison diluted and strangled the mother-in-law several minutes after she drank it, to make sure that she was dead. The perpetrator had an unclear psychiatric background. The family attorney tried in vain to raise an insanity defence, but since she was never admitted to a psychiatric facility, the appointed forensic experts excluded any major psychopathology that could have led to the crime. However, some further details, disclosed by witnesses during the trial, revealed her fragile social status and interpersonal relations.

The perpetrator had been sent several times from her relatives (mostly from her sister) to a self-proclaimed medicine man with 'supranatural' healing abilities, and had some "hypnosis sessions" in his office. The medicine man was not certified for such activities, and his practices of divination and healing were not serious or professional. The offered treatment provided few, if any, improvement in the psychic status of the perpetrator.

Finally, the case was brought to the court of law, and she was charged with first-degree murder. Life imprisonment was requested by the state prosecutor. However, pregnancy and birth of her child was considered as a mitigating factor to the jury. She was sentenced to 32 years of imprisonment, with the verdict waiting confirmation in a court of appeal.

DISCUSSION

Family relations are highly vulnerable when there are no compensatory mechanisms for altercations and everyday quarrels. In our case, the perpetrator gave her mother-in-law a poisoned drink, pretending that it was a miraculous drink that could appease them; and at the end she strangulated the victim. The pregnant woman in our case, while complaining of her previous miscarriage, was convinced that magic was responsible for it. Meanwhile, she told her husband that, "I feel there is a rabbit in my abdomen", while notifying him of another pregnancy. Among her personal belongings, forensic experts found a file from the Internet, downloaded on her USB flash drive, named "The Killing of the Magician". The objects (meat, animal teeth, human hair *etc.*) allegedly found close to her bedroom or inside it, and which she believed to be witchcraft supplies, are also normal parts of the armamentarium of a sorcerer.

The main question while facing a thought distortion within the context of magical thinking, remains as to whether this phenomenon and magic itself should be considered as superstitions, popular beliefs, or both. Culturally accepted deviations, that in other settings are considered as an abnormal behaviour and therefore not accepted, will not always be granted the status of a mental disorder. Sorcery-related violence and/or magic remains a significant social phenomenon in Papua New Guinea, Indonesia, several African countries, and Canada, to mention a few[13-16]. The social attempt to end these occurrences and face them legally has led to the promulgation of several laws. The Anglo-Saxon tradition is rich in witchcraft acts and has been adopted step by step in different countries, including the colonies of the British Empire. Some acts of the Scottish Parliament had even considered death penalty ("Anentis Witchcraftis") in 1563, leading to the murder of 2000 people after being sentenced for sorcery in the century to come[17]. Therefore, this issue has significant social and psychological impacts, causing societies to pendulate between the strong innate belief in supernatural powers and the ad hoc attempts to influence or at least modulate such seemingly out of control powers. This has led to deterministic evaluations of human behaviour as a whole in some theories of psychopathology[18].

Efforts to approach under a psychopathological perspective the acts of criminal violence involving witchcraft or sorcery are obviously warranted[10,19]. Philological

contributions are of particular assistance, especially from a psychodynamic/psychoanalytical point of view. Freud pioneered a milestone work, entitled "Animismus, Magie und Allmacht der Gedanken" (Animism, Magic and Omnipotence of Thought) in his "Totem and Taboo" book[20]. Melvin-Koushki while commenting recently on the work of Günther and Pielow, differentiated a variety of beliefs (Glaube) in German: (1) "Aberglaube" (superstition); (2) "Unglaube" (disbelief); (3) "Irrglaube" (misbelief); and (4) "Volksglaube" (folk belief)[21].

The criminologists' need for the expertise of psychiatric professionals might have reversed the assumption that heinous felonies are perpetrated only from mentally ill persons. Meanwhile, forensic psychiatry has distanced itself from its root disciplines, and while adopting a criminological terminology, one should consider the reasons for such an aberration. However, this might not be only related to terminological choices, albeit they alone can be corruptive[22]. While summoning psychiatrists in court not unusually judges raise questions on why and how the perpetrator acted: This is a trap that produces aberrating answers. Such an issue, before being a psychiatric dilemma, is a criminological concept that will lead back to pre-classical period of theories, with the devil and demonic possessions responsible of all wrongdoings[23].

Belief in magic is not only restricted to underdeveloped or suburban areas, but economic hardship also will fuel its popularity. It seems that people still resort to magic, witchcraft, and sorcery as a medium when they feel a situation is out of their control or beyond logical explanations. For example, some tribes still occasionally use magic while fishing in open seas, but not in safe lagoons[24]. The reason for the use of witchcraft may be as simple as the individual's attempt to influence things and conditions that are out of control; such choices resemble obsessive ruminations. However, DSM has included magical thinking not only among schizophrenic disorders, but also within a larger spectrum of personality disorders[10]. Recently, straightforward relationships have been suggested between magical thinking and hallucinations, although again in a schizophrenic background[25].

A research published in PubMed, with the keywords: "Witchcraft", "magic", and "forensic psychiatry", yielded approximately 24 papers, which is a minuscule figure when compared to the total number of published papers in medicine. Therefore, it is important to expand research in this area to enrich the findings. It is as well possible to integrate other fields of study, such as anthropology, ethnology, philology, and other related studies. It seems that magical thinking has survived in affiliation to delusional disorders, although it is strictly considered only for clinical psychiatric or psychological purposes. However, in courts of law, the belief in magic as a psychopathological phenomenon is regularly rejected, whenever an insanity defense or mental illness are proposed as mitigating factors. Attempts to connect sorcery and magic as being prevalent within some specific religious confessions have been ever since dismissed. In fact, the cultural osmosis and the globalization have universalized even these mental health issues and concerns[26].

CONCLUSION

The legal authorities will have the final say in such complicated cases, with long-term effects on the families. Magic and spelling objects (witchcraft) are rarely object of the prosecutors' investigations, which may be related to the public abhorrence on witchcraft, or as an attempt to prevent notorious trials from happening again, such as in the case of Salem and its aftermath[3]. Generally, experts, including toxicologists, coroners, and psychiatrists, are an important part of investigations[27].

The concept of evil, once considered as outdated, is resurging in philology, theology, forensic psychiatry, and other scientific areas[22,28-30]. The background of the expertise might differ: A criminologist will offer a diverse perspective from a forensic psychiatrist. If an overlapping occurs, this is mainly due to the unlucky exchanging of roles, in an era where authorities are downplaying the value of psychiatric opinions thus predicting their demise in criminal courts[31]. Adopting a criminological terminology is therefore not helpful to psychiatrists. Focusing on psychiatric phenomenology and remaining as descriptive as one can be while reporting a case, is the keystone of a mindful and convincing expertise[32]. This is even more tangible when the situations involve an intricate mesh of anthropological, social and psychological issues such as when dealing with belief in magic, witchcraft and crime perpetrated under the influence of a delusional state.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 51-53

DOI: 10.13105/wjma.v9.i1.51

ISSN 2308-3840 (online)

MINIREVIEWS

Should we use full analgesic dose of opioids for organ procurement in brainstem dead?

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Author contributions: Charlier C wrote the first draft of the manuscript with significant critical input from Rebibo JD and Benmoussa N.

Conflict-of-interest statement: None for any of all authors.

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Manuscript source: Invited manuscript

Specialty type: Medical ethics

Country/Territory of origin: France

Peer-review report's scientific quality classification

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Abstract

Families facing the growing demand for organ removal from their loved ones are questioning the possible suffering of the brainstem dead patient. A frequent question they ask to coordinating doctors is: Are you sure he will not feel anything? Currently due to the risk of exacerbation of spinal reflexes and abnormal movements following surgical stimuli, it is recommended to use a curarization and an analgesic agent (most often morphine). The doses of opioids are less important than during usual anaesthesia, whereas the person is considered brainstem dead and there is no longer any cerebral integration of the pain. But what assures us that absolutely no more sensibility exists at this precise moment? Should the use of full analgesic dose of opioids not be continued anyway? Could this make the levies more "ethical"?

Key Words: Bioethics; Transplantation; Anaesthesiology; Brainstem dead

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Core Tip: Families facing the growing demand for organ removal from their loved ones are questioning the possible suffering of the brainstem dead patient. A frequent question they ask to coordinating doctors is: Are you sure he will not feel anything? Currently due to the risk of exacerbation of spinal reflexes and abnormal movements following surgical stimuli, it is recommended to use a curarization and an analgesic agent (most often morphine). The doses of opioids are less important than during usual anaesthesia, whereas the person is considered brainstem dead and there is no longer

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 27, 2020
Peer-review started: December 27, 2020
First decision: January 25, 2021
Revised: January 30, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Cheungpasitporn W, Tai MCT, Xia VW
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH

any cerebral integration of the pain. But what assures us that absolutely no more sensibility exists at this precise moment? We propose that further neuroscientific analyses be conducted in order to improve our knowledge about such a sensibility. We ask to the medical community if we should rather use full analgesic dose of opioids, and question if this would make the levies more "ethical"?

Citation: Charlier P, Rebibo JD, Benmoussa N. Should we use full analgesic dose of opioids for organ procurement in brainstem dead? *World J Meta-Anal* 2021; 9(1): 51-53
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/51.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.51

INTRODUCTION

The definition of death is changing, driven by clinical physicians, physiologists and biomedical ethics specialists[1]. This evolution, both semantic and diagnostic, seems to be completely independent of religious influences, whether monotheistic or not. Recently, the concept of brain death has been called into question[2], with this underlying idea (and certainly provocative): What if the first motivation behind the introduction of this patho-physiological concept was not the extended possibility of access to graft organs, rather than death itself[3]?

ETHICAL AND PHYSIOLOGICAL CONSIDERATIONS

With this moving and fluctuating definition of death (the moment of death, but also the stage of death)[4], we see that the definition of a total insensitivity of the deceased is debated: It may not be so immediate, nor so complete as initially considered. In the context of the most ethical medical and surgical practice possible, particularly in the context of critical care medicine, it is legitimate to wonder about a possible evolution of our professional practices.

Families facing the growing demand for organ removal from their loved ones are questioning the possible suffering of the brainstem dead patient. A frequent question they ask to coordinating doctors is: Are you sure he will not feel anything? Currently due to the risk of exacerbation of spinal reflexes and abnormal movements following surgical stimuli, it is recommended to use a curarization and an analgesic agent (most often morphine). The doses of opioids are less important than during usual anaesthesia[5], whereas the person is considered brainstem dead and there is no longer any cerebral integration of the pain. In this context, what assures us that absolutely no more sensibility exists at this precise moment? Should the use of full analgesic dose of opioids not be continued anyway? Could this make the levies more “ethical”?

CONCLUSION

The distress of the families is understandable, but the administration of analgesics at usual dose could also create a confusion of the families and be a source of misunderstanding between the declaration of death of their relative and the use of analgesic during the procedure of organ extraction[6]. This question should be taken into account by an international college of anaesthesiologists and bioethics specialists. In addition, it is likely that neurosensory experiments are necessary, not only at the level of the cerebral stage, but also of the spinal cord.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 54-63

DOI: 10.13105/wjma.v9.i1.54

ISSN 2308-3840 (online)

MINIREVIEWS

Risk factors, manifestations, diagnosis and treatment of cholelithiasis in children

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Author contributions: Xu ZR collected and analyzed literature and wrote the paper; Dan HL contributed literature analysis and arrangement; Yu F designed the research study; and all authors have read and approve the final manuscript.

Conflict-of-interest statement: All authors have no conflict of interest, financial or otherwise.

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Manuscript source: Unsolicited manuscript

Specialty type: Behavioral sciences

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Abstract

Cholelithiasis in children refers to cholelithiasis or choledocholithiasis in newborns, infants and adolescents, including a series of symptoms and complications related to stones. The incidence rate of cholelithiasis in children is far lower than that in adults. However, the detection rate of cholelithiasis in children has risen in recent years. The aetiology of cholelithiasis in children is complex, and the pathogenesis is still unclear. According to existing studies, cholelithiasis in children may be related to sex and hormones, obesity, drugs, haemolytic diseases, genetic factors, chemokines, abnormal biliary tract formation, ileal surgery, total parenteral nutrition and other factors. In addition, there is a large occurrence of idiopathic cholelithiasis in children with unknown aetiology. The clinical manifestations of cholelithiasis in children are not completely the same as those in adults, mainly due to the different ages and causes. At present, there is no consensus on the clinical treatment of cholelithiasis in children. Follow-up and observation, drug dissolution and surgical treatment are all treatment methods for children with cholelithiasis. This study reviewed the related literature reports of cholelithiasis in children and summarized the risk factors, clinical manifestations, diagnosis and treatment of cholelithiasis in children to improve the understanding, diagnosis, and treatment of cholelithiasis in children.

Key Words: Cholelithiasis; Children; Risk factors; Diagnosis; Treatment

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Core Tip: The aetiology of cholelithiasis in children is complex, which may be related to sex and hormones, obesity, drugs, haemolytic diseases, genetic factors, chemokines, abnormal biliary tract formation, ileal surgery, total parenteral nutrition and other factors. The clinical manifestations of cholelithiasis in children are not completely the

Country/Territory of origin: China

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 19, 2021
Peer-review started: January 19, 2021
First decision: February 14, 2021
Revised: February 18, 2021
Accepted: February 22, 2021
Article in press: February 22, 2021
Published online: February 28, 2021

P-Reviewer: Tashkandi E
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH

same as those in adults, mainly due to the different ages and causes. The treatment of cholelithiasis in children is individualized. Follow-up and observation, drug dissolution and surgical treatment are all treatment methods for children with cholelithiasis.

Citation: Xu ZR, Dan HL, Yu F. Risk factors, manifestations, diagnosis and treatment of cholelithiasis in children. *World J Meta-Anal* 2021; 9(1): 54-63
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/54.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.54

INTRODUCTION

In recent years, the incidence rate of cholelithiasis in children has increased[1-4]. According to a recent literature review[5], the incidence of cholecystectomy in children under the age of 16 in England increased from 0.78 in 1997 to 2.7 per 100000 in 2012. The incidence of cholecystectomy in subjects under 18 years old increased from 8.8 per 100000 person-years in 1993 to 13.0 per 100000 person-years in 2012, and a 9-year retrospective study in the United States ending in 2012 also showed that the incidence of cholecystectomy in children had increased by 216%. The incidence rate of cholelithiasis in children under 19 years old is 0.13%-2%[6,7], but it is still lower than the incidence of cholelithiasis in adults. The pathogenesis of cholelithiasis in children is not clear, and there is no uniform standard for treatment. Its complications or surgical consequences may affect children's social adaptability and healthy growth. Therefore, cholelithiasis in children has attracted increasing attention.

RISK FACTORS FOR CHOLELITHIASIS IN CHILDREN

Gallstone formation is secondary to local factors (*i.e.*, impaired smooth muscle function, wall inflammation and intraluminal mucin accumulation) and bile (*i.e.*, cholesterol supersaturation and solid crystal precipitation) and "extra-gallbladder" features, such as genetic polymorphisms, epigenetic factors, hormonal factors, multilevel alterations in cholesterol metabolism, altered intestinal motility, and variations in gut microbiota[5]. What are the characteristics of the causes of cholelithiasis in children? Early studies mostly indicate that there is a large proportion of children with cholelithiasis from blood system diseases. With the accumulation of cases and increased research, cholelithiasis in children is also related to many factors, such as total parenteral nutrition, ileal disease and long-term fasting[2,8-10]. Among the risk factors for cholelithiasis in children, haemolytic factors account for 20%-30%, and other known causes account for 40%-50% (including total parenteral nutrition, ileal diseases, congenital biliary diseases, *etc.*). Other idiopathic cholelithiasis accounts for 30%-40% of cases[11]. In the infant stage, cholelithiasis often does not have a clear predisposing factor. In the childhood stage, the onset of cholelithiasis is often associated with chronic haemolytic disease or ileal disease. In the adolescent stage, idiopathic cholelithiasis accounts for the majority of cases, which is similar to adult cholelithiasis[6]. Risk factors for cholelithiasis in children are shown in Table 1.

Sex and oestrogen

Female patients are more common in children with cholelithiasis[12]. One study from Turkey showed that among 254 children with cholelithiasis with an average age of 8.9 ± 5.2 years, the proportion of female patients was 52.8%[13]. Another study reported that women accounted for 63.8% of children with cholelithiasis diagnosed at Bialystok Medical University within 4 years[14]. To a large extent, this imbalance is due to the significant increase in the proportion of adolescent women with gallstones related to oestrogen. There are key enzymes involved in oestrogen inactivation and activation and the expression of oestrogen receptors α and β in normal gallbladder mucosa, which regulates the concentration of oestrogen in the human gallbladder[15]. The increase in oestrogen levels may lead to stone formation by increasing the secretion of cholesterol and reducing the synthesis of bile acid[16]. Overall, the incidence of gallstones in women is 2 times that of men. The increase in oestrogen levels during pregnancy will lead to significant metabolic changes in the liver and gallbladder

Table 1 Risk factors for cholelithiasis in children

Serial number	Risk factors	
1	Sex and oestrogen	
2	Obesity	
3	Drugs	
4	Haemolytic diseases	
5	Genetic factors	
6	Other known factors	Family history of cholelithiasis
		Chemokines
		Abnormal biliary tract formation
		Biliary ascariasis
		Ileal surgery
		Total parenteral nutrition
7	Idiopathic cholelithiasis	

system, including the formation of cholesterol supersaturation and slow gallbladder motion. These two factors promote gallstone formation[17]. Through the activation of 5-β-hydroxylase, oestrogen in adolescence can increase the synthesis of cholesterol, which makes supersaturated cholesterol precipitate in the bile to form cholesterol stones, which is the main risk factor for the increased prevalence of gallstones in adolescent women.

Obesity

In a comparative study on the body mass index (BMI) of children with and without cholelithiasis, the median age of 98 children with simple cholelithiasis (75 cases) and children with common bile duct stones (23 cases) was 16 years old. The BMI of children with simple cholelithiasis and choledocholithiasis was significantly higher than that of the normal control group, and the BMI of children with choledocholithiasis was significantly higher than that of children with simple cholelithiasis[18]. Therefore, it can be seen that an increase in BMI is related to the occurrence of cholelithiasis or choledocholithiasis in children, and obesity is a risk factor for cholelithiasis and choledocholithiasis.

Drugs (ceftriaxone sodium)

Ceftriaxone sodium is an anion that can be 20 to 150 times more concentrated in bile than in serum. It easily forms insoluble salt (ceftriaxone calcium) with calcium and precipitates in the gallbladder. Because cholelithiasis caused by ceftriaxone is reversible and disappears after stopping treatment, it is called "pseudolithiasis". In different prospective studies, the incidence of ceftriaxone-induced pseudolithiasis ranged from 15% to 46%[13]. Ceftriaxone is a commonly used antibiotic for the treatment of severe bacterial infections in paediatric hospitals. Cholelithiasis or cholestasis is more common in children than in adults. A review of ceftriaxone-associated cholelithiasis in children suggests that ceftriaxone's relation to cholelithiasis is dose-dependent[19].

Haemolytic diseases

Studies have shown that 39% of children with cholelithiasis have haemolytic disease[7]. A study conducted at the Universidade de Campinas found a higher incidence (45%) of cholelithiasis in patients with sickle cell disease (SCD) and a predominance of younger patients (< 15 years)[20]. The incidence rate of cholelithiasis in children with SCD is significantly higher due to increased hembilirubin levels and promotion of gallstone formation due to haemolysis[21-24]. SCD is a common cause of cholelithiasis. A total of 261 SCD children aged 4 mo to 16 years from the Children's Emergency Hospital in Khartoum were followed up for 13 years. The total prevalence of cholelithiasis was 11.5% (30/261) and increased with age[23]. Karayalcin *et al*[25] diagnosed cholelithiasis in 47 children aged 2-18 years with cholelithiasis using oral cholecystography and cholecystography, and the prevalence of cholelithiasis was 17%

(8/47). In another study using abdominal ultrasound, 305 children with SCD with an average age of 10.45 years were screened for cholelithiasis, and the prevalence of cholelithiasis was 19.7%. A correlation was found between the occurrence of stones and increased children's age[26]. In Jos, northern Nigeria, a prospective gallstone prevalence study was conducted in 65 children with SCD aged 5-13 years by abdominal ultrasound. The results showed that the prevalence of gallstones was 4.6%[27]. The low prevalence of cholelithiasis in SCD patients in Senegal may be due to the tolerance of Senegalese haplotypes. Older children with SCD and severe chronic haemolysis are the main causes of cholelithiasis in children in Senegal[28].

Genetic factors

Studies have confirmed that genetic variation in the UGT1A promoter region has a significant impact on serum bilirubin levels and symptomatic cholelithiasis in children with SCD[29,30]. A study on the association between genetic factors and other factors [including haematological parameters, clinical severity, hydroxyurea (HU) treatment] and cholelithiasis in children with SCD suggested that genetic polymorphisms of UGT1A1 (TA) were not the only cause of cholelithiasis in children with SCD, and its occurrence was also affected by low basal reticulocyte RET count, number of alpha gene deletions, HU treatment, and frequency of vascular occlusion events[21]. In addition, the incidence rate of cholelithiasis in children with hereditary spherocytosis (HS) is also high[30], and the incidence rate is reported to be as high as 41%[31]. Therefore, it is recommended that HS children start physical examinations every year at the age of 4. Gilbert's syndrome, as an autosomal dominant genetic disease, increases indirect bilirubin in the liver due to insufficient glucuronosyltransferase activity in liver microsomes, thus increasing the risk of cholelithiasis[30,31]. Moreover, a recent study showed that the risk trait of cholesterol metabolism in childhood favouring cholesterol gallstone disease later in adulthood is influenced by risk variant 19H of ABCG8[32]. The occurrence of cholelithiasis in children is related to genetic factors or genetic diseases.

Other known factors

Any disease that can cause changes in the structure and function of the biliary system may become part of the aetiology or a potential risk factor for cholelithiasis in children. For example, gallbladder fibrosis caused by chronic inflammation, chronic active hepatitis and other liver infections or metabolic diseases, pregnancy and postpartum pregnancy is accompanied by high levels of oestrogen and progesterone. Blood transfusion haemolysis can also increase the incidence of gallstones.

Family history of cholelithiasis: A Turkish study on cholelithiasis in children shows that the most common risk factor for cholelithiasis in children is a family history of cholelithiasis[33]. Similar conclusions have also been found in other studies[7,34]. In Frybova *et al*[18]'s study, 24% of children with cholelithiasis had a family history of cholelithiasis.

Chemokines: A prospective study carried out by Zdanowicz *et al*[35] showed that the serum level of chemokines in 54 non-obese children and adolescents with cholelithiasis was significantly higher than that of 26 healthy people of the same age, suggesting that chemokines may play a potential role in the development of cholelithiasis in children and adolescents.

Abnormal biliary tract formation: Some scholars believe that cholelithiasis in children can be caused by abnormal formation of the biliary tract[36]. Congenital biliary dysplasia can cause stricture or dilatation of intrahepatic and extrahepatic bile ducts, cholestasis complicated with bacterial infection, and gradual gallstone formation.

Biliary ascariasis: Biliary ascariasis can not only cause bile duct obstruction and mucosal damage but also form stones as the core. In addition, the E. coli introduced by ascariasis lumbricoides also produces β-glucuronidase, which hydrolyses soluble conjugated bilirubin into unconjugated bilirubin and precipitates with calcium ions to form bilirubin stones.

Ileal surgery: Because 98% of bile salt is reabsorbed at the end of the ileum, resection of ileum or ileal lesions can interrupt the intestinal and hepatic circulation of bile salt, resulting in a relative increase in cholesterol and precipitation from bile to form stones. The incidence rate of cholelithiasis in patients undergoing ileostomy is 24.5%, which is 3 times that of normal people. In patients with an ileal resection over 10 cm, the incidence of cholelithiasis is significantly higher[37]. However, there was no

cholelithiasis in the paediatric group in this study, which may be related to the continued growth of children's intestinal tract.

Total parenteral nutrition: Total parenteral nutrition (TPN) is commonly used in the treatment of neonatal congenital malformations, necrotizing enterocolitis, severe malnutrition, premature infants and other situations. However, TPN may damage intestinal and hepatic circulation and the role of cholecystokinin, resulting in bile stasis, bile sludge and stone formation[38]. The longer the duration of TPN treatment, the higher the risk of cholelithiasis. With ileum resection or ileal disease, the risk of long-term TPN cholelithiasis in children is increased[39], and the same is true for newborns. The incidence of bile sludge in newborns treated with TPN is significantly increased[40].

Idiopathic cholelithiasis in children

In addition to the abovementioned risk factors, the causes of cholelithiasis in many children are unknown with no clear risk factors before onset. This issue is called idiopathic cholelithiasis in children. A study of cholelithiasis in children showed that 56.6% if cases in 254 children with cholelithiasis (mean age: 8.9 ± 5.2 years) were idiopathic[13]. Similarly, in a study on the efficacy of laparoscopic surgery for cholelithiasis in children, only 5 (27.8%) of the 18 children had definite aetiological risk factors for cholelithiasis, and the remaining 13 (75.2%) had idiopathic cholelithiasis[41]. A study from Turkey also showed that no risk factor for stone formation was encountered in 50% of 70 children with cholelithiasis[42].

MANIFESTATIONS AND DIAGNOSIS

Pigment stones are the most common type of gallstones in children[41]. However, in adolescents, idiopathic cholesterol gallstones account for the majority of stone, which is similar to adults. At the same time, cholecystolithiasis accounted for 80%-90% of cases, and choledocholithiasis accounted for 10%-20% of cases[6]. Abdominal pain is the most common symptom of cholelithiasis[13,43], and often manifests as right upper abdominal colic or abdominal distension pain, which is also the situation in most children with cholelithiasis. In a retrospective study from Pittsburgh Children's Hospital, 197 children with symptomatic cholelithiasis (age 13.89 ± 0.26) had abdominal pain for 3.52 ± 0.56 mo, followed by nausea and vomiting (33.0%), weight loss (3.6%), diarrhoea (2.5%), abdominal distension (0.5%) and constipation (0.5%)[44]. However, several special types of cholelithiasis in children are usually asymptomatic, such as ceftriaxone-associated cholelithiasis. Only in a few cases may abdominal pain, nausea and vomiting occur. When complications from ceftriaxone-related cholelithiasis are suspected, ultrasound examination should be performed[19]. In addition, children with sickle cell disease are often asymptomatic when they are diagnosed with cholelithiasis. A 13-year follow-up study of 26 children with SCD cholelithiasis from Sudan showed that only one patient developed symptoms 3 years later and received surgical treatment. The remaining 25 patients had no symptoms of cholelithiasis[23]. Cholelithiasis in children can also cause complications. It has been reported that the incidence of cholecystitis, choledocholithiasis and acute pancreatitis was 27.7%, 10.6% and 23.4%, respectively. In the differential diagnosis of abdominal pain, even the youngest child should pay attention to cholelithiasis, especially premature infants, low-birth-weight infants and very-low-birth-weight infants[14]. In symptomatic cholelithiasis in children, 47.9% of the patients were complicated with cholecystitis by pathological examination after laparoscopic cholecystectomy[45].

Other diseases of the gallbladder often cause abdominal pain, which is easily confused with cholelithiasis in children, such as chronic cholecystitis, biliary ascariasis, and biliary dyskinesia. Among 1611 patients aged 9-17 who underwent LC identified from the 2012 to 2013 American College of Surgeons' National Surgical Quality Improvement Program Paediatrics data, the most frequent indications for surgery were cholelithiasis/biliary colic (34.3%), chronic cholecystitis (26.9%), and biliary dyskinesia (18.2%)[44]. Biliary dyskinesia is one of the main differential diagnoses of cholelithiasis in children. It attributes intermittent right upper abdominal pain to abnormalities in gallbladder function[46]. Ultrasound is the first choice for the diagnosis of cholelithiasis in children. It is not only relatively simple but also easy to obtain the child's cooperation. In a follow-up study of 185 children with SCD conducted in Trousseau Children's Hospital (Paris), 26 cases of cholelithiasis were examined by ultrasonography, and abdominal ultrasonography was recommended for patients

under 7 years old with sickle cell anaemia[24]. A Senegalese study also recommended systematic abdominal ultrasound at least once a year in patients with SCD over the age of five[28]. It was recommended that repeated ultrasonography be used to observe gallstone formation in children with high-risk factors for gallstone disease for at least 10 years[47]. When common bile duct stones are found, it is necessary to exclude choledochal cysts with abnormal connections of the pancreaticobiliary duct. Magnetic resonance cholangiopancreatography should be performed first[6].

THERAPEUTIC TACTICS

The treatment strategy for cholelithiasis in children is a particularly difficult problem. Some scholars believe that different treatment methods can be adopted for different age groups[4,48].

Observation

For asymptomatic cholelithiasis in children, especially in newborns and infants, an observation method suitable for individuals should be adopted according to the underlying disease and the degree of illness, including follow-up, regular clinical data collection, regular ultrasound or blood test. The duration of observation should also be heterogeneous, ranging from months to years and even to adulthood in some children. One study reported that 82% of children with atypical symptoms of gallstones improved through diet control and had no complications during an average follow-up period of 21 mo. This study concluded that children with asymptomatic or atypical symptoms could be safely followed up[49]. Some children's cholelithiasis manifests in the form of bile sludge, which is often caused by long-term fasting (especially during total parenteral nutrition). After the patients resume an oral diet, the sludge and gallstones may disappear[50].

Bile acid dissolution therapy

Ursodeoxycholic acid (UDCA) is controversial in the treatment of cholelithiasis because it is not effective at preventing symptoms and complications that subsequently occur as there is a high long-term recurrence rate[50]. UDCA can significantly reduce cholesterol saturation by 40%-60% by inhibiting the absorption and secretion of cholesterol sterols. In adults, clinical studies have shown that the stone dissolution rate is 30%-60%[33]. Some studies believe that UDCA in the treatment of children with gallstones, in addition to alleviating symptoms, has no obvious effect[51]. Most children responded to UDCA treatment in the first six months, but children with haemolytic diseases showed no response to UDCA. Therefore, UDCA treatment may be useful before surgery in asymptomatic patients with cholelithiasis without haemolytic diseases[33]. The dissolution rate of cholelithiasis was higher in younger children and those with biliary sludge formation and ceftriaxone-related cholelithiasis but lower in older children and haemolytic, anaemia-related cholelithiasis[13]. Ceftriaxone-associated cholelithiasis gradually disappeared after withdrawal of ceftriaxone for a period of time (days to months). Therefore, conservative treatment and long-term follow-up are recommended[19]. In a study by Serdaroglu *et al*[42], the results showed that ursodeoxycholic acid had no effect on stone dissolution, but diagnosis under 2 years old, having a single stone and small stone size (less than 5 mm) were influential factors.

Extracorporeal shock wave lithotripsy

As early as the 1980s and 1990s, studies showed that gallstone disease may be treated successfully and without serious adverse effects by extracorporeally generated shock waves in selected patients[52,53]. Evidence from randomized controlled trials, systematic reviews and cohort studies shows that extracorporeal shock wave lithotripsy, similar to bile acid dissolution therapy with UDCA alone, has a low cure rate[50].

Surgical therapy

According to European Association for the Study of the Liver Clinical Practice Guidelines on the prevention, diagnosis and therapy of gallstones, cholecystectomy is recommended for symptomatic gallbladder stones but not routinely for asymptomatic gallbladder stones. However, asymptomatic patients with porcelain gallbladder may undergo cholecystectomy[50]. Surgical decisions in children with cholelithiasis are more complicated. In a comparative study of cholelithiasis and biliary sludge in children, the authors suggest that cholecystectomy should not be routinely performed in

children with cholelithiasis or sludge but should be performed after careful selection of patients at risk for complications[7]. With laparoscopic cholecystectomy (LC) becoming the gold standard for the treatment of gallstones, an elective LC procedure is recommended for cholelithiasis in children with SCD to prevent potential complications of biliary colics, acute cholecystitis, and choledocholithiasis[54-57]. LC is a safe and effective method for the treatment of cholelithiasis in children[22], and obesity is an independent risk factor for increased operative time in children undergoing LC[44]. In addition, cholecystolithotomy is used in the treatment of cholelithiasis in children, and 14 of the 15 patients were not symptomatic with normal echography controls[58]. Minimally invasive cholecystolithotomy using laparoscopy combined with choledochoscopy is a safe and viable technique, which can be successfully applied in pediatric surgery on the premise of mastering the indications, and has incomparable advantages over LC[59]. Treatment of secondary common bile duct stones includes interventional radiologic, endoscopic or surgical procedures. Stone extraction may be performed with endoscopic retrograde cholangiopancreatography with or without sphincterotomy combined with LC[6]. The sequential approach of endoscopic sphincterotomy and stone extraction followed by LC is a safe and effective approach for the management of cholelithiasis and choledocholithiasis in children[60].

CONCLUSION

Cholelithiasis in children should be paid more attention in clinic. The aetiology of cholelithiasis in children is complex, which may be related to sex and hormones, obesity, drugs, haemolytic diseases, genetic factors, chemokines, abnormal biliary tract formation, ileal surgery, total parenteral nutrition and other factors. The clinical manifestations of cholelithiasis in children are not completely the same as those in adults, mainly due to the different ages and causes. The management of cholelithiasis in children is individualized. Follow-up and observation, drug dissolution and surgical treatment are all treatment methods for children with cholelithiasis, which can be individualized according to different ages and causes of onset. Through the review of the etiology, diagnosis and treatment of cholelithiasis in children, we can distinguish it from adult cholelithiasis, which will help us to improve the understanding and management of cholelithiasis in children.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 64-73

DOI: 10.13105/wjma.v9.i1.64

ISSN 2308-3840 (online)

SYSTEMATIC REVIEWS

Mortality of critical care interventions in the COVID-19: A systematic review

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Author contributions: Davis J conceived and designed the project, initiated the search, assisted in data analysis, drafted the manuscript, and made critical revisions for content to the manuscript; Leff R, Patel A and Venkatesan S assisted in data collection, data analysis, and made critical revisions for content to the manuscript; and all authors have given final approval for the manuscript to be published and agree to be accountable for all aspects of the work.

Conflict-of-interest statement: To the best of our knowledge, the authors have no conflicts of interest, financial or other, to declare regarding this manuscript.

PRISMA 2009 Checklist statement: The analysis presented was conducted in alignment with PRISMA 2009 guidelines and a copy of the checklist is available from the authors.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in

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Abstract

BACKGROUND

The novel coronavirus severe acute respiratory syndrome coronavirus 2 is associated with a severe disease known as coronavirus disease 2019 (COVID-19). A small percentage of patients with COVID-19 will require intensive care and possibly mechanical ventilation. The mortality of intensive care interventions in patients with COVID-19 remains unclear.

AIM

To identify mortality rate of COVID-19 patients receiving different interventions in the critical care unit.

METHODS

We searched OVID Medline, SCOPUS, MedRxIv, preprints.org, and Centers for Disease Control databases from November 2019 to April 10, 2020 for articles on COVID-19. Teams of 2 independent reviewers reviewed titles and abstract for studies that reported mortality of human adults with COVID-19 and exposure to a critical care intervention [Intensive care admission, mechanical ventilation, acute hemodialysis, extracorporeal membrane oxygenation, or cardiopulmonary resuscitation (CPR)]. We used a descriptive analysis and unweighted averages of mortality across studies.

RESULTS

Our search identified 6973 articles and 20 met inclusion: 17 for intensive care, 13 for mechanical ventilation, 5 for hemodialysis, 2 for extracorporeal membrane

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Manuscript source: Invited manuscript

Specialty type: Health care sciences and services

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 20, 2021
Peer-review started: January 20, 2021
First decision: February 14, 2021
Revised: February 16, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Fan Y, Kurniawan A
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH



oxygenation, and 1 for CPR. Mortality associated with intensive care admission ranged from 9%-83%, with overall mortality 32.5% (95%CI: 32.4%-32.6%). Mortality associated with intubation from 16.7%-100% with overall mortality 64.0% (95%CI: 62.4%-65.5%). In patients requiring hemodialysis, mortality ranged from 0%-100%, with average mortality 75.3% (95%CI: 72.6%-77.4%).

CONCLUSION

Patients with COVID-19 requiring intensive care have high mortality rates. Authorities can use this data to establish pharmacoeconomic studies to make decisions about allocation of scarce resources if necessary.

Key Words: Coronavirus; SARS-CoV-2; COVID-19; Mortality; Systematic review; Critical care

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Core Tip: This systematic review identifies the mortality associated with critical care interventions in patients with novel coronavirus disease 2019. These include intensive care admission, mechanical ventilation, acute hemodialysis, extracorporeal membrane oxygenation, or cardiopulmonary resuscitation. Average mortality for intensive care admission was 32.5%. While mortality is high, this is lower than initially described in early reports of the pandemic. Physicians can hopefully use this data to inform decisions about goals of care or, if necessary, allocation of scarce resources.

Citation: Davis J, Leff R, Patel A, Venkatesan S. Mortality of critical care interventions in the COVID-19: A systematic review. *World J Meta-Anal* 2021; 9(1): 64-73
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/64.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.64

INTRODUCTION

The novel coronavirus disease 2019 (COVID-19) has caused an international pandemic. The pandemic nature of this virus is largely attributed to its virulence and high mortality. Initial reports had mortality as high as 2% in China[1]; 4% in Wuhan, China (the site of initial outbreak)[2]; and 6% in Italy. Later data with widespread testing placed case fatality rate estimates closer to 1%[3]. Nonetheless, another major issue in the pandemic nature of this virus is its ability to overwhelm healthcare systems, particularly the critical care needs of healthcare systems[4]. This includes intensive care beds, ventilators, and dialysis units and was particularly evident in "hot spot" areas, for example Lombardy, Italy and New York City, New York early in the pandemic and many other areas since.

Patients in critical care have mortality rates as high as 50%[5], and patients who require mechanical ventilation have mortality as high as 80%[6]. This is higher than typical patients with viral pneumonia or acute respiratory distress syndrome, which has mortality closer to 35%-45%[7]. Mortality with COVID-19 is much higher in the elderly[3] and those with comorbid conditions, hypertension, diabetes, obesity, and heart disease[8,9]. However, there have been young cases, without any preexisting medical conditions, who required mechanical ventilation and died[10]. Further, patients admitted to intensive care have markedly long lengths of stay in the hospital, on the order of weeks to months[11].

This overwhelming burden on healthcare systems has led to unprecedented discussions about rationing of care[4] and universal Do Not Resuscitate orders[12-14]. These discussions arise in order to preserve precious healthcare resources in overburdened systems as well as to protect healthcare workers from increased exposure to potentially aerosolizing procedures like cardiopulmonary resuscitation, intubation, or bag ventilation. If interventions were universally futile, the decision to not perform them might be easier.

Nonetheless, a small percentage of patients do survive intensive care admission and mechanical ventilation. There are even case reports of patients surviving

extracorporeal membrane oxygenation (ECMO)[15-17] and cardiopulmonary resuscitation (CPR)[18]. Thus, these interventions, when implemented in the correct patient population, can save lives.

In order to help inform decisions and discussions about critical care interventions in patients with COVID-19, the authors sought to perform a systematic review of the mortality rate of COVID-19 patients receiving different interventions in the critical care unit.

MATERIALS AND METHODS

This study had no human subjects and was, therefore, exempt from review by th Institutional Review Board Review. This study was conducted in accordance with Preferred Reporting in Systematic Review and Meta-Analysis Guidelines (PRISMA)[19,20]. The PRISMA Checklist is available in Supplement and the research protocol is available from the authors upon request.

Search strategy and study selection

We searched OVID Medline, SCOPUS, MedRxIv, preprints.org, and Centers for Disease Control (CDC) databases from November 2019 to April 10, 2020. The search used keyword terms for "COVID-19", "SARS", and "coronavirus". The CDC database was already compiled and was only filtered for English articles. An example search strategy is available in Supplementary material. We also reviewed citations from included articles, review articles, and suggestions from select content experts to find relevant articles that may have been missed. Titles and abstracts from these search methods were screened independently by 3 trained reviewers (Leff R, Patel A, Venkatesan S). If any reviewer thought an article was potentially relevant, a full text copy of the article was ordered and again reviewed for inclusion by these 3 authors. Inclusion criteria were studies that evaluated human adult patients with COVID-19 and exposure to a critical care intervention, defined as either (1) intensive care admission; (2) endotracheal intubation or mechanical ventilation; (3) acute hemodialysis or renal replacement; (4) ECMO; or (5) CPR. Exclusion criteria included opinions, abstracts, non-English papers, studies on children, studies with < 5 participants, or studies with not enough data to address the research question. We did plan to contact authors for updated data if data was incomplete. All authors independently identified articles for exclusion and any discrepancies were resolved with a consensus meeting among all 4 authors.

Data extraction and data synthesis

After title and abstract selection, a standardized abstraction form was developed by all of the authors. Two of three authors (Leff R, Patel A, Venkatesan S) abstracted data from the studies and compared their results for discrepancies. These were resolved by consensus among all authors. Data included author, publication year, population, setting, intervention, mortality, and other outcomes.

Statistical analysis

Data were tracked in Microsoft Excel® (Seattle, WA, United States). Inter-rater reliability was assessed using accuracy and Cohen's kappa. We used the Newcastle-Ottawa Scale to assess risk of bias in the studies[21]. Given the observational nature of these studies without intervention, heterogeneity was not formally assessed. It is assumed to be high. Where applicable, simple meta-analysis was performed using raw, unweighted percentages.

RESULTS

We identified 6973 articles in our search and 20 met inclusion criteria[7,18,22-39] [17 for Intensive care unit (ICU) care[7,22-37], 13 for mechanical ventilation[7,23,24,29-32,34-39], 5 for hemodialysis[24,29,33,36,37], 2 for ECMO[30,36], and 1 for CPR[19], Figure 1]. Details of each study are presented in Table 1. We attempted to reach all authors for updated data and received responses from one author[31]. We also found an updated publication of the report from International Care National Audit and Research Centre (ICNARC) and used the May 1st version of this report[29]. All of the studies were published in 2020, with 15 from Asia[18,23-25,27,28,30-38], 2 from Europe[26,29], 2 from the United States[7,22], and one

Table 1 Studies included in a systematic review of mortality and critical care interventions in coronavirus disease 2019

Ref.	Data available					Newcastle-Ottawa quality scale
	Intensive care	Mechanical ventilation	Hemodialysis	ECMO	CPR	
Bhatraju *et al*[6]	X	X				4
Shao *et al*[18]					X	6
Arentz *et al*[22]	X					4
Cai *et al*[23]	X	X				6
Cao *et al*[24]	X	X	X			6
Chen *et al*[25]	X					5
Grasselli *et al*[26]	X					5
Han *et al*[27]	X					7
Huang *et al*[28]	X					7
Intensive Care National Audit and Research Centre[29]	X	X	X			5
Li *et al*[30]	X	X		X		4
Ling *et al*[31]	X	X				6
Wang *et al*[32]	X	X				7
Wang *et al*[33]	X		X			7
Wang *et al*[34]	X	X				6
Wu *et al*[35]	X	X				6
Yang *et al*[36]	X	X	X	X		7
Zhou *et al*[37]	X	X	X			7
Deng *et al*[38]		X				6
Grein *et al*[39]		X				5

ECMO: Extracoropreal membrane oxygenation; CPR: Cardiopulmonary resuscitation.

study which included multiple regions[39]. The ICNARC data was the largest reported cohort and greatly overwhelmed other studies' sample size. Given this and that overall heterogeneity was subjectively high and there was a low number of studies, meta-analysis was not performed for any intervention other than ICU admission or intubation/mechanical ventilation. Quality scoring revealed generally moderate quality observational studies (Table 1). The accuracy among two selectors was 81%, and interrater reliability for selection was moderate at $\kappa = 0.63$ (95%CI: 0.39-0.87).

ICU admission

The 17 studies on mortality in COVID-19 patients requiring ICU admission included 9828 patients[7,22-37]. Mortality associated with ICU admission ranged from 9%[25] to 83%[36], with overall mortality of 32.5% (3198/9828, 95%CI: 32.4%-32.6%). However, it is important to note that several studies still had a significant number of patients in the hospital or ICU or did not report long term outcomes. Of studies that reported discharge to home, 242/401 (60.3%. 95%CI: 55.5%-65.1%) patients are clearly reported to be discharged home (Table 2). As mentioned, the ICNARC data is the largest sample and showed a mortality rate of 33% and contributed 7542 of the 9828 patients. The ICNARC sample only reports patients discharged from critical care (2642/7542, 35.0%, 95%CI: 33.9%-36.1%), and not long-term disposition to home. The mortality was similar when excluding ICNARC data (701/2286, 30.7%, 95%CI: 28.8%-32.6%).

Endotracheal intubation/mechanical ventilation

The 13 studies on mortality in COVID-19 patients requiring mechanical ventilation included 3865 patients[7,23,24,29-32,34-39]. The mortality ranged from 16.7%[31] to 100%[38] with

Table 2 Mortality associated with intensive care admission in coronavirus disease 2019

Ref.	Died	Sent home	Total patients	Mortality
Bhatraju *et al*[6]	12	5	24	50.0%
Arentz *et al*[22]	11		21	52.4%
Cai *et al*[23]	3		30	10.0%
Cao *et al*[24]	6	12	18	33.3%
Chen *et al*[25]	2		22	9.1%
Grasselli *et al*[26]	405		1581	25.6%
Han *et al*[27]	3	12	15	20.0%
Huang *et al*[28]	5	7	13	38.5%
Intensive Care National Audit and Research Centre[29]	2497		7542	33.1%
Li *et al*[30]	4		8	50.0%
Ling *et al*[31]	1	1	8	12.5%
Wang *et al*[32]	6	9	36	16.7%
Wang *et al*[33]	7		11	63.6%
Wang *et al*[34]	133	185	344	38.7%
Wu *et al*[35]	44		53	83.0%
Yang *et al*[36]	20		52	38.5%
Zhou *et al*[37]	39	11	50	78.0%
Total	3198	242	9828	32.5% (average)

a mean of 64.1% (2422/3787, 95%CI: 62.4%-65.5%) (Table 3). Many patients remain intubated or in the ICU. Of studies reporting it, there were 14% (32/322, 95%CI: 10.6%-18.2%) of intubated patients who were eventually discharged to home. Again, the ICNARC data contributed 3,508 of these patients with a mortality of 62.0% (2175/3508, 95%CI: 63.6%-60.4%). The ICNARC data for this category only reports patient with an outcome of discharged from the ICU or death. Patient still receiving mechanical ventilation and long-term dispositions are not reported in this data. When not including the ICNARC data, the average mortality was 77.1% (262/340, 95%CI: 72.6%-81.6%).

Hemodialysis/renal replacement

There were 5 studies of 1203 patients that reported outcomes of patient who received acute hemodialysis, which included continuous renal replacement therapy or intermittent hemodialysis[24,29,33,36,37] (Table 4). Mortality ranged from 0%[33] to 100%[37], with the average mortality being 75.3% (898/1193, 95%CI: 72.6%-77.4%), with ICNARC contributing 1163 of these patients and the ICNARC mortality being 75% (870/1163, 95%CI: 72.5%-77.5%)[29]. Mortality of studies not including ICNARC data was 80% (32/40, 95%CI: 92.4%-67.3%). Of the remaining 8 patients not in the ICNARC data, 7 were discharged from the hospital and one was discharged from the ICU.

ECMO

Only 2 studies met our criteria for reporting more than 5 patients who received ECMO[30,36]. In these studies, the mortality was 50% (4/8)[30] in one and 83% (5/6)[36] in the other. Li *et al*[30] report of the remaining 4 patients, 3 were taken off ECMO and one is still on ECMO, and Yang *et al*[36] report their surviving patient to be discharged from the hospital. Several other studies included in our analysis report 100% mortality for small numbers of patients on ECMO: 1[35], 2[38], and 3[37] patients. Cao *et al*[24] report that of their 3 patients, 1 died and 2 were discharged from the hospital, and Grein *et al*[39] report that of their 4 patients, 1 was discharged home and 3 remain on ECMO.

CPR

Only 1 study reported of significant sample size on cardiac arrest[18]. In this study of 136 patients who received CPR in the hospital, ROSC was achieved in 18 (13.2%,

Table 3 Mortality associated with endotracheal intubation in coronavirus disease 2019

Ref.	Died	Total patients	Mortality
Bhatraju *et al*[6]	9	18	50.0%
Cai *et al*[23]	3	30	10.0%
Cao *et al*[24]	12	14	33.3%
Intensive Care National Audit and Research Centre[29]	2175	3508	33.1%
Li *et al*[30]	4	8	50.0%
Ling *et al*[31]	1	6	12.5%
Wang *et al*[32]	6	17	35.2%
Wang *et al*[34]	97	10	38.7%
Wu *et al*[35]	44	67	83.0%
Yang *et al*[36]	19	22	38.5%
Zhou *et al*[37]	31	32	78.0%
Deng *et al*[38]	21	21	100.00%
Grein *et al*[39]	6	34	17.6%
Total	2428	3787	64.1% (average)

Table 4 Mortality associated with hemodialysis in coronavirus disease 2019

Ref.	Died	Total patients	Mortality
Cao *et al*[24]	5	6	83.3%
Intensive Care National Audit and Research Centre[29]	870	1163	74.8%
Wang *et al*[33]	5	5	100.0%
Yang *et al*[36]	8	9	88.9%
Zhou *et al*[37]	10	10	100.0%
Total	898	1193	75.3% (average)

95%CI: 7.5%-18.9%) patients, of which 4 (2.9%, 95%CI: 0%-5.7%) patients were still alive at 30 days, and 1 was reported to have a good neurologic outcome (0.7%, 95%CI: 0%-2.1%) with a cerebral performance category score of 1 or 2.

DISCUSSION

Our study is the first systematic review on outcomes in critical care patients with COVID-19. We showed an average ICU mortality of 33% and mortality of intubated patients of 64%. The mortality rates we found are less than those initially reported out of China[2,36], but higher than similar reports of patients with acute respiratory distress syndrome or viral pneumonia[8]. Other, more intensive interventions (Hemodialysis, ECMO, and CPR) were associated with increasing mortality.

Given the nature and spread of COVID-19, healthcare resources have been overwhelmed. This has led some institutions to discuss appropriate allocation of these scarce resources (*i.e.*, ventilators, ICU beds, healthcare personnel). Many long-term care facilities and hospitals have placed an appropriate emphasis on advanced directives among those patients most at risk for severe disease. Our data should help physicians quantify outcomes associated with various critical care interventions.

Some hospitals have controversially instituted universal "Do Not Resuscitate" orders in order to conserve resources and protect healthcare resources[12-14]. We only identified one study on a large data set of patients receiving CPR. This showed only 1/136 patients (0.7%) who required CPR survived neurologically intact. This supports the concept that CPR is largely, though not universally futile. Given that there is also risk to healthcare workers, institutions should proactively work to identify which

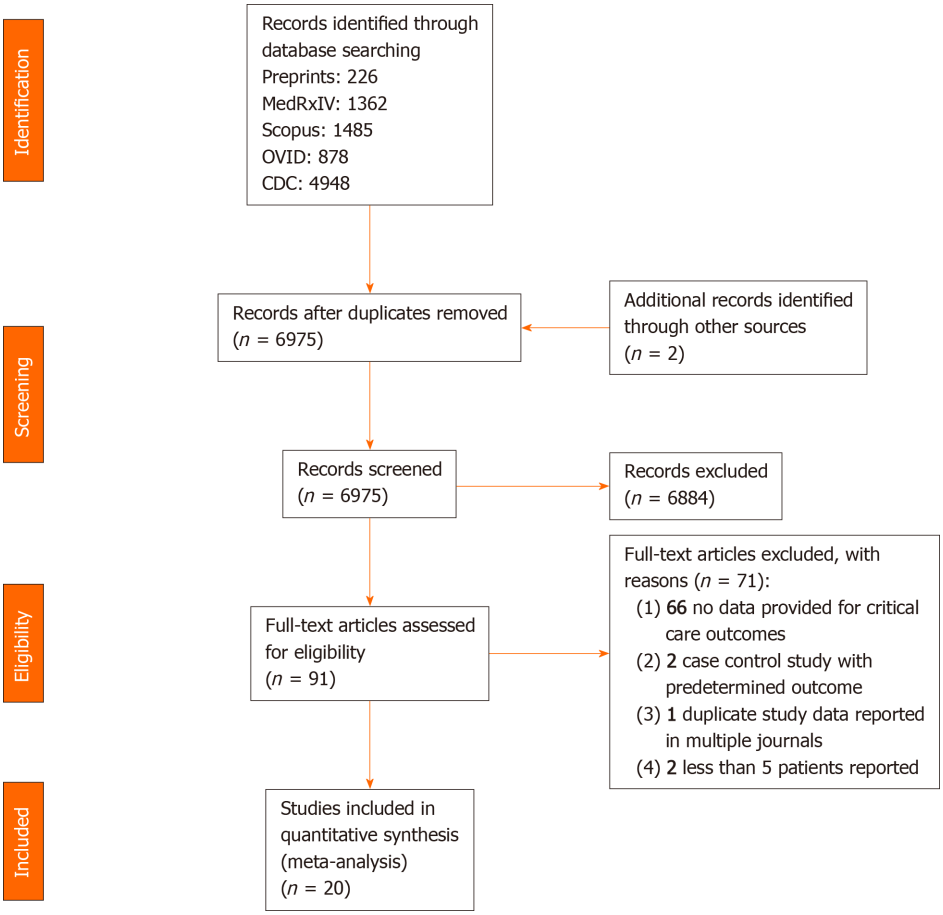


Figure 1 The Preferred Reporting in Systematic Review and Meta-Analysis Guidelines flow diagram for a systematic review of mortality and critical care interventions in coronavirus disease 2019.

patients with COVID-19 would benefit from CPR. For example, ventilated patients who suffer hypoxic arrest probably have little to gain from CPR, unless it is a bridge to some other form of oxygenation like ECMO.

Of course, decisions regarding patient care and limitations must also be personalized to individual patients. Unfortunately, given the summary nature of our data, it cannot be used to express a patient's individual risk. This will be based on many factors, including age and comorbidities which are not included in our analysis. Mortality alone is also not the only prognostic outcome of interest to many patients. Further, beyond prognosis, decisions regarding care limitations must also consider a patient's functional status and preferences and values.

One of the most important limitations of our analysis is that many patients remained in the ICU at the time of this analysis. This undoubtedly leads to incomplete estimates of mortality. Of the studies that reported data, we attempted to include data on patients discharged from the hospital, as well. We also attempted to reach authors for updated data in this regard, but only received a response from one author. There have also been reported concerns regarding duplicate publications of data, which could lead to overrepresentation of some samples[40]. Some analyses are influenced by a few large data sets (*i.e.*, ICNARC), but it is reassuring that mortality estimated remain nearly the same when not including this data. Finally, the data on COVID-19 continues to rapidly evolve, and treatment paradigms continue to change. Therefore, it is possible that mortality may improve as treatments improve.

CONCLUSION

In conclusion, COVID-19 is a novel disease process, which is associated with high mortality in those patients requiring critical care. The intensity of intervention seems to be associated with increasing mortality. Clinicians should consider this data, along with patient-specific factors, when deciding which intensive care interventions to

initiate. Complete data considering disposition of all patients will improve our understanding of the prognosis of this new disease process.

ARTICLE HIGHLIGHTS

Research background

The novel coronavirus disease 2019 (COVID-19) has ravaged humans across the globe. Mortality is high, especially in the most critically ill patients. Due to the pandemic nature of the virus, some locations have been forced to ration resources.

Research motivation

Critical care is, by nature, highly resource intensive. With limited resources and an overwhelming number of critically ill patients in some communities, discussions about rationing limited resources have begun. Fundamental to these discussions is understanding the probability of survival of the most intensive interventions.

Research objectives

Our objective was to identify, *via* systematic review, the mortality rate of COVID-19 patients receiving different interventions in the critical care unit, namely intensive care admission, mechanical ventilation, acute hemodialysis, extracorporeal membrane oxygenation, or cardiopulmonary resuscitation.

Research methods

We conducted a systematic review of several databases using accepted systematic review methods to identify the mortality rate of COVID-19 patients receiving intensive care admission, mechanical ventilation, acute hemodialysis, extracorporeal membrane oxygenation (ECMO), or cardiopulmonary resuscitation (CPR). Studies included were observational.

Research results

Our search identified 20 studies overall. Mortality associated with intensive care admission averaged 32.5%. Mortality associated with intubation averaged 64.0%. In patients requiring hemodialysis, mortality averaged 75.3%. There were limited studies on ECMO and CPR. The one study on CPR showed only 0.7% (1/136) had a good neurologic outcome.

Research conclusions

Mortality among COVID-19 patients requiring intensive care is high, though not as high as initially reported. This information can be used to guide goals of care of individual patients and system level discussion about rationing scarce resources when locales are overwhelmed.

Research perspectives

Critical care does have a high, but certainly not universal, mortality rate. Further research should identify individual risk factors for poor outcome among patients requiring critical care. CPR, though, appears to have low chance of survival. The decision to perform CPR on critically ill patients should be very carefully considered.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 February 28; 9(1): 74-87

DOI: 10.13105/wjma.v9.i1.74

ISSN 2308-3840 (online)

META-ANALYSIS

Efficacy and safety outcomes with remdesivir in COVID-19 patients: A meta-analysis

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Conflict-of-interest statement: All authors declare having no conflicts of interest.

PRISMA 2009 Checklist statement: Study has been conducted as per the PRISMA 2009 checklist.

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Abstract

BACKGROUND

Remdesivir is a broad-spectrum antiviral drug having in vitro activity against severe acute respiratory syndrome coronavirus 2 and is currently being used on a compassionate basis outside of clinical trials.

AIM

To analyze the efficacy and safety of remdesivir compared with other interventions in coronavirus disease 2019 (COVID-19) patients.

METHODS

We searched online databases to include randomized controlled trials evaluating the efficacy and safety of remdesivir compared with other interventions in COVID-19 patients. We summarized efficacy and safety data as risk ratios (RRs) with 95% confidence interval (CI) and used Mantel-Haenszel fixed or random-effect models. We estimated the number needed to treat (NNT) to cause one additional outcome. We used the GRADE approach to assess the quality of the evidence for all outcome parameters.

RESULTS

We included four randomized controlled trials. We observed no significant difference in mortality (RR: 0.83; 95%CI: 0.57–1.20; I^2 = 59%) and rate of ventilation (RR: 0.69; 95%CI: 0.41-1.18; I^2 = 77%) between remdesivir- and placebo-treated patients. Remdesivir showed higher rates of clinical recovery than placebo (RR: 1.10; 95%CI: 1.04–1.16; I^2 = 0%; NNT: 14.3). We observed no difference in overall adverse events between remdesivir- and placebo-treated patients (RR: 1.05;

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Manuscript source: Invited manuscript

Specialty type: Infectious diseases

Country/Territory of origin: India

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: November 12, 2020
Peer-review started: November 12, 2020
First decision: February 12, 2021
Revised: February 16, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Martinez MA, Şehirli AÖ
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li X

95%CI: 0.86–1.27; I^2 = 77%). We observed less risk of serious adverse events (RR: 0.75; 95%CI: 0.63–0.89; I^2 = 0%) in remdesivir- than placebo-treated patients. The GRADE approach suggested moderate quality of evidence for all efficacy and safety outcomes.

CONCLUSION
We observed limited clinical benefit of remdesivir over placebo in the treatment of COVID-19. Our findings could be biased because of the small number of trials.

Key Words: COVID-19; SARS-CoV-2; Antiviral; Pneumonia; Remdesivir; Meta-analysis; Systematic review

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Core Tip: Remdesivir is an antiviral drug having in vitro and in vivo activity against severe acute respiratory syndrome coronavirus 2. United States Food and Drug Administration has granted emergency use authorization for its use in all hospitalized patients. The findings of this meta-analysis suggest remdesivir can enhance clinical recovery without having significant benefits on mortality outcomes. because of lack of any major safety concerns, its use is recommended in the absence of effective antiviral drugs against coronavirus disease 2019.

Citation: Patel TK, Patel PB, Barvaliya M, Vijayalaxmi, Bhalla HL. Efficacy and safety outcomes with remdesivir in COVID-19 patients: A meta-analysis. *World J Meta-Anal* 2021; 9(1): 74-87
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/74.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.74

INTRODUCTION

The novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first described in China. Later it was declared a pandemic by the World Health Organization (WHO). COVID-19 is highly contagious, and is associated with significant morbidity and mortality[1-4]. Currently, there are no definitive proven antiviral treatments for COVID-19. Several potential therapeutic options, including remdesivir, have been evaluated for the treatment of COVID-19[5].

Remdesivir is a broad-spectrum antiviral drug having activity against several RNA viruses, including filoviruses (*e.g.*, Ebola virus, Marburg virus), coronaviruses (*e.g.*, SARS-CoV, Middle East respiratory syndrome coronavirus, and paramyxoviruses (*e.g.*, respiratory syncytial virus, Nipah virus, and Hendra virus). It is a prodrug metabolized within cells into the active nucleoside triphosphate. This nucleoside triphosphate acts as an analog of adenosine triphosphate (ATP) and competes with the natural ATP substrates to selectively inhibit RNA-dependent RNA polymerase resulting in delayed RNA chain termination during the process of viral replication[6,7]. Delayed chain termination at positions I and 3 are key elements of inhibition observed with SARS-CoV, MERS-CoV[6] and SARS-CoV-2 RNA-dependent RNA polymerase complexes[8,9]. An in vitro study by Wang *et al*[10] suggested that remdesivir could achieve a therapeutic working concentration against SARS-CoV-2 in Vero E6 cells. It inhibited virus infection in human liver cancer Huh-7 cells, which are sensitive to SARS-CoV-2[10]. Moreover, prophylactic and therapeutic administration of remdesivir reduced the virus replication, disease severity and lung damage caused by MERS-CoV in the rhesus macaque animal model[11].

The clinical evidence of utility of remdesivir in COVID-19 patients is limited. It is primarily used on a compassionate basis outside of clinical trials in the absence of other available effective treatment options[12,13]. Recently published studies have shown contradictory findings about the efficacy of remdesivir in COVID-19. The adaptive COVID-19 treatment trial (ACTT) trial suggested better clinical recovery and trends of mortality reduction with remdesivir[14], while he WHO solidarity trial did not find a

significant reduction in mortality with the use of remdesivir in COVID-19[15]. In this meta-analysis, we aimed to assess the efficacy and safety of remdesivir in COVID-19 patients based on evidence from published randomized controlled clinical trials.

MATERIALS AND METHODS

Literature search

We searched the clinical studies of remdesivir in COVID-19 patients in PubMed, medrxiv.org, biorxiv.org, mediterranee-infection.com/pre-prints-ihu, LILACS, CNKI and Google Scholar. The PubMed search terms were: ("Remdesivir") AND ("COVID-19" OR "SARS-CoV-2" OR "Coronavirus"). The last search was run on October 28, 2020. There were no language restrictions for inclusion of the studies.

Types of participants

Patients of any age and either sex who had virologically confirmed SARS-CoV-2 infection were included in the analysis.

Types of studies

Inclusion criteria: (1) Randomized controlled clinical trials; (2) Open labeled or blinded studies; and (3) Comparative trials of remdesivir with any other interventions including placebo.

Exclusion criteria: (1) Studies of remdesivir other than SARS-CoV-2; (2) Observational studies, noncomparative studies, case reports; (3) In vitro and animal studies of remdesivir in COVID-19; and (4) Review articles, commentaries, viewpoints, or editorials.

Types of intervention

Use of remdesivir in COVID-19 patients irrespective of dose and duration of therapy was considered. All treatment modalities including placebo were considered as the comparator arm.

Risk of bias assessment

The methodological characteristics of included studies were assessed through five domains of the revised Cochrane risk of bias assessment tool for randomized controlled clinical trials (ROB-II)[16].

Data extraction

We imported the data into a Microsoft Excel 2016 spreadsheet. The extracted data included publication details, study design, study site, demographics of the study population, baseline clinical characteristics, remdesivir dose, duration and mode of administration, comparator, supportive care, and outcome variables (*i.e.* number of deaths, number of patients requiring invasive ventilation, clinical recovery and serious adverse events).

Types of outcome measures

Efficacy outcomes: The efficacy outcome variables were the RRs of cumulative mortality, composite mortality and ventilation, mechanical ventilation or extracorporeal membrane oxygenation ECMO) rate and clinical recovery rate of remdesivir in comparison with other interventions. In case of multiple time-point estimation of outcomes in the included studies, we used data at the end of study periods. Intention to treat data were used to estimate the efficacy outcomes.

Safety outcomes: The safety outcome variables were participants with adverse events (overall, grade 3 or 4, and serious adverse events) in the remdesivir and comparator arms. The safety population was used to estimate the safety outcomes. All patients who received remdesivir and comparator drugs irrespective of per-protocol dose and duration were considered as a safety population and included in the estimation of safety outcomes.

Data synthesis and meta-analysis

All outcomes were dichotomous variables. They were summarized as a risk ratio (RR) with 95% confidence interval (CI) using the Mantel-Haenszel method with a fixed- or

random-effect model. The selection of meta-analysis model was based on the presence of heterogeneity. The fixed model effect was preferred in the absence of heterogeneity. Heterogeneity was estimated through I^2. Sensitivity analysis of efficacy and safety outcomes was performed based on risk of bias assessment and study design. The meta-analytic summary was calculated by excluding studies showing "some concern" or high risk of bias as per the ROB-II tool and those with an open-label design.

The pooled risk difference of meta-analytic summary was estimated. It was used to calculate the number needed to treat (NNT) to cause one additional outcome[17]. The GRADE approach was used to analyze the quality of the evidence for each of the efficacy and safety outcomes. It was assessed based on the following parameters: study limitations, inconsistency, indirectness of evidence, imprecision, and publication bias[18,19]. The meta-analysis was conducted with Review Manager version 5.4.1.

RESULTS

Of the 2289 retrieved references, we selected 46 publications for full-text evaluation (Figure 1). We included four randomized controlled trials analyzing the effects of remdesivir in COVID-19 patients.

Characteristics of included studies

Table 1 shows the general characteristics of the included studies. The included studies used remdesivir as an add-on to the standard care of treatment. Beigel *et al*[14] conducted a double-blind, randomized, placebo-controlled, multicenter study in North America, Europe and Asia; 541 patients were assigned to the remdesivir and 521 to the placebo arms. The treatment arms were comparable at baseline for age, gender, race, median time from symptom onset to randomization, co-morbid conditions and severity status.

The WHO Solidarity Trial Consortium *et al*[15] was a solidarity trial conducted by the WHO. It was a randomized, open label, parallel arm, multicenter study investigating the effect of four repurposed medications (remdesivir, hydroxychloroquine, interferon-β1a, and lopinavir/ritonavir) with local standard care. The comparator of remdesivir was a group of patients with a similar probability of allocation to receive remdesivir, but instead receiving standard care. A total of 2743 patients were assigned to receive remdesivir. The corresponding 2708 patients were assigned to receive local standard care. Both treatment arms were comparable at baseline for age, gender, co-morbidity, bilateral lung lesions, respiratory support and prior impatient days before randomization.

Spinner *et al*[20] conducted a randomized, open-label, placebo-controlled, multicenter study in moderate COVID-19 pneumonia patients at North America, Europe and Asia. A total of 197 patients were assigned to receive remdesivir of 10 d duration, 199 to remdesivir of 5 d duration and 200 to placebo. As all other included studies administered remdesivir for 10 d, we used remdesivir data of 10 d treatment duration only. The treatment arms (remdesivir 10 d and placebo) were comparable at baseline for age, gender, race, co-morbidity, and duration of symptoms before the administration of interventions and duration of hospitalization before the administration of interventions. The placebo-treated group had a higher percentage of patients with baseline oxygen requirement (19% *vs* 13%). Compared with the remdesivir-treated group, a higher percentage of patients in the placebo arm received hydroxychloroquine/chloroquine (45% *vs* 11%), lopinavir/ritonavir (22% *vs* 6%) and azithromycin (31% *vs* 21%).

Wang *et al*[21] conducted a double-blind, randomized, placebo-controlled, multicenter study in China. A total of 158 patients were assigned to receive remdesivir and 78 to receive placebo. Both treatment arms were comparable at baseline for age, gender, body temperature, viral load, oxygen therapy support and co-interventions (interferon alfa-2b, lopinavir/ritonavir or corticosteroid administration). The remdesivir-treated group had a higher percentage of patients with co-morbidities and a faster respiratory rate at baseline. The placebo-treated group had a higher percentage of patients with early symptom onset at baseline.

Risk of bias in included studies

Three trials were considered to have a low risk of bias for all the domains of the ROB-II tool (randomization process, effect of assignment to intervention, effect of adhering to intervention, missing outcome data, measurement of the outcome, selection of the reported results and overall risk of bias assessment). Spinner *et al*[20] was considered to

Table 1 General and baseline characteristics of included studies

Reference	Design	Study population	Total number of participants randomized/ completed	Age, mean ± SD years/median (IQR)	Male gender, %	Coexisting conditions, %	Severity status, %	Drug, dose and duration of interventions		Follow-up duration in d
								Remdesivir	Comparator	
Beigel *et al*[14]	Double-blind, randomized, placebo-controlled trial	Hospitalized COVID-19 patients with evidence of lower respiratory tract involvement	Total: 1062/1025; Remdesivir: 541/517; Placebo: 521/508	Remdesivir: 58.6 ± 14.6; Placebo: 59.2 ± 15.4	Total: 64.4; Remdesivir: 65.1; Placebo: 63.7	Remdesivir: DM (30.8) HT (50.6); Placebo: DM (30.4); HT (50.9)	Remdesivir: MV or ECMO (24.2); Placebo: MV or ECMO (29.6)	Day 1: 200 mg iv loading dose on day 2-10: 100 mg iv daily	Placebo	29
WHO Solidarity Trial Consortium *et al*[15]	Open-labeled, randomized controlled Trial	Hospitalized COVID-19 patients	Remdesivir: 2750/2743; SC: 2725/2708	Remdesivir: NS; SC: NS	Total: 62.9; Remdesivir: 62.2; SC: 63.7	Remdesivir: DM (25.8); HD (20.8); Asthma (5.1); SC: DM (24.6); HD (20.9); Asthma (5.1)	Remdesivir: Ventilated (9.2); SC: Ventilated (8.6)	Day 1: 200 mg iv loading dose on day 2-10: 100 mg iv daily	SC	28
Spinner *et al*[20]	Open-labeled, randomized controlled Trial	Hospitalized moderate severity COVID-19 pneumonia patients	Total: 397/393; Remdesivir: 197/193; SC: 200/200	Remdesivir: 56 (45-66)[1]; SC: 57 (45-66)[1]	Remdesivir: 61; SC: 63	Remdesivir: DM (44); HD (58); HT (44); Asthma (16); SC: HT (41); Asthma (14); DM (38); HD (54);	Remdesivir: Supplemental oxygen (13); SC: Supplemental oxygen (19)	Day 1: 200 mg iv loading dose on day 2-10: 100 mg iv daily	SC	28
Wang *et al*[21]	Double-blind, randomized, placebo-controlled trial	Severe COVID-19 with pneumonia	Total: 237/226; Remdesivir: 158/150; Placebo: 79/76	Test: 66 (57-73)[1]; Placebo: 64 (53-70)[1]	Remdesivir: 56; Placebo: 65	Remdesivir: DM (25); HT (46); HD (9); Placebo: DM (21); HT (38); HD (3)	Remdesivir: Supplemental oxygen (100); Placebo: Supplemental oxygen (95)	Day 1: 200 mg iv loading dose on day 2-10: 100 mg iv daily	Placebo	28

[1]Median (interquartile range). All patients received either remdesivir or placebo as an add-on to supportive care. DM: Diabetes mellitus; ECMO: Extracorporeal membrane oxygenation; HD: Heart disease; HT: Hypertension; IQR: Interquartile range; MV: Mechanical ventilation; NS: Not specified; SC: Supportive care; WHO: World Health Organization.

have some concerns for the effect of assignment to intervention domain because of imbalance in co-interventions among remdesivir- and placebo-treated patients. Hence, the overall risk of bias was considered to have some concern as per ROB-II tool (Table 2).

Meta-analytic summary of efficacy outcomes

Mortality: As shown in Figure 2A, we observed no significant difference in the RR of mortality between remdesivir and control patients (RR: 0.83; 95%CI: 0.57–1.20). An I^2 = 59% suggested a moderate degree of between-trial heterogeneity. The NNT to cause one additional reduction in mortality of COVID-19 patients with remdesivir was 100 (95%CI: −25–100). The GRADE approach suggested moderate quality evidence (Table 3). The sensitivity analysis showed a similar trend in mortality (Table 4).

Ventilation: As shown in Figure 2B, we did not observe any significant difference in RR of mechanical ventilation or ECMO between remdesivir and placebo or supportive care treatment (RR: 0.69; 95%CI: 0.41–1.18; I^2 = 77%) (Figure 2B). The NNT to cause one

Table 2 Risk of bias assessment as per the Revised Cochrane risk of bias tool for randomized trials

Domain No.	Risk of bias domains	Beigel *et al*[14]	WHO Solidarity Trial Consortium *et al*[15]	Spinner *et al*[20]	Wang *et al*[21]
1	Risk of bias arising from the randomization process	Low	Low	Low	Low
2a	Risk of bias due to deviations from the intended interventions (effect of assignment to intervention)	Low	Low	Some concern	Low
2b	Risk of bias due to deviations from the intended interventions (effect of adhering to intervention)	Low	Low	Low	Low
3	Missing outcome data	Low	Low	Low	Low
4	Risk of bias in measurement of the outcome	Low	Low	Low	Low
5	Risk of bias in selection of the reported result	Low	Low	Low	Low
--	Overall risk of bias assessment	Low	Low	Some concerns	Low

WHO: World Health Organization.

Table 3 Quality assessment for efficacy and safety parameters as per the GRADE approach

No. of studies (design)	Limitation	Inconsistency	Indirectness	Imprecision	Publication bias	Quality	RR (95%CI)
Mortality							
Four (RCT)	No serious limitations	Serious inconsistency (I^2 = 59%)	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	0.83 (0.57-1.20)
Ventilation							
Four (RCT)	No serious limitations	Serious inconsistency (I^2 = 77%)	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	0.69 (0.41-1.18)
Composite Mortality and ventilation							
Four (RCT)	No serious limitations	Serious inconsistency (I^2 = 78%)	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	0.80 (0.58-1.11)
Clinical recovery							
Three (RCT)	No serious limitations	No serious inconsistency	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	1.10 (1.04-1.16)
Adverse event							
Three (RCT)	No serious limitations	Serious inconsistency (I^2 = 77%)	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	1.05 (0.86-1.27)
Grade 3 or 4 adverse event							
Three (RCT)	No serious limitations	No serious inconsistency	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	0.88 (0.79-0.99)
Serious adverse event							
Three (RCT)	No serious limitations	No serious inconsistency	No serious indirectness	No serious imprecision	Asymmetric Funnel plot	Moderate	0.75 (0.63-0.89)

CI: Confidence interval; RCT: Randomized controlled trial; RR: Risk ratio.

additional reduction in need of ventilation of COVID-19 patients with remdesivir was 50 (95%CI: −16.7–100). The GRADE approach suggested moderate quality evidence (Table 3). The sensitivity analysis based on study design suggested a trend of benefit with the use of remdesivir (RR: 0.57; 95%CI: 0.410.77; I^2 = 0%).

Composite mortality and ventilation: We observed no benefit of remdesivir in reducing the risk of the composite outcome of mortality and ventilation compared with placebo or supportive care (RR: 0.80; 95%CI: 0.58–1.11; I^2 = 78%) (Figure 2C). This

Table 4 Sensitivity analysis of efficacy and safety outcomes based on the risk of bias assessment and study design

Variable	All studies		Sensitivity analysis(risk of bias assessment)		Sensitivity analysis (study design)	
	RR (95%CI)	I^2	RR (95%CI)	I^2	RR (95%CI)	I^2
Mortality	0.83 (0.57-1.20)	59	0.83 (0.55-1.26)	73	0.74 (0.38-1.44)	65
Ventilation	0.69 (0.41-1.18)	77	0.73 (0.42-1.27)	83	0.57 (0.41-0.77)	00
Composite mortality and ventilation	0.80 (0.58-1.11)	78	0.83 (0.60-1.16)	84	0.69 (0.55-0.86)	10
Clinical recovery	1.10 (1.04-1.16)	00	1.10 (1.02-1.18)	00	1.10 (1.02-1.18)	00
Adverse event	1.05 (0.86-1.27)	77	0.94 (0.86-1.02)	00	0.94 (0.86-1.02)	00
Grade 3 or 4 adverse event	0.89 (0.80-0.99)	00	0.87 (0.71-1.07)	10	0.87 (0.71-1.07)	10
Serious adverse event	0.75 (0.63-0.90)	00	0.76 (0.64-0.92)	00	0.76 (0.64-0.92)	00

CI: Confidence interval; RR: Risk ratio.

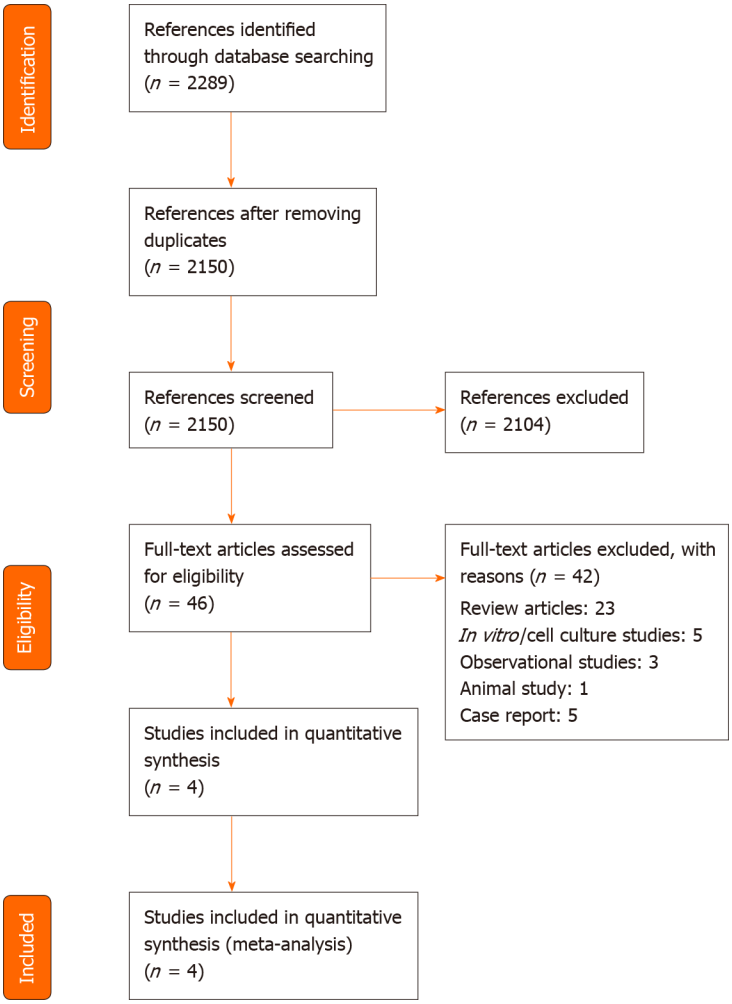


Figure 1 Study selection – preferred reporting items for systematic reviews and meta-analysis flow diagram.

corresponds to an NNT to cause one additional participant to experience mortality and ventilation in COVID-19 patients receiving remdesivir compared with placebo of 25 (95%CI: −12.5–100). The GRADE approach suggested moderate quality evidence (Table 3). The sensitivity analysis based on study design suggested a trend of benefit in composite outcome with the use of remdesivir (RR: 0.69; 95%CI: 0.55–0.86; I^2 = 10%) (Table 4).

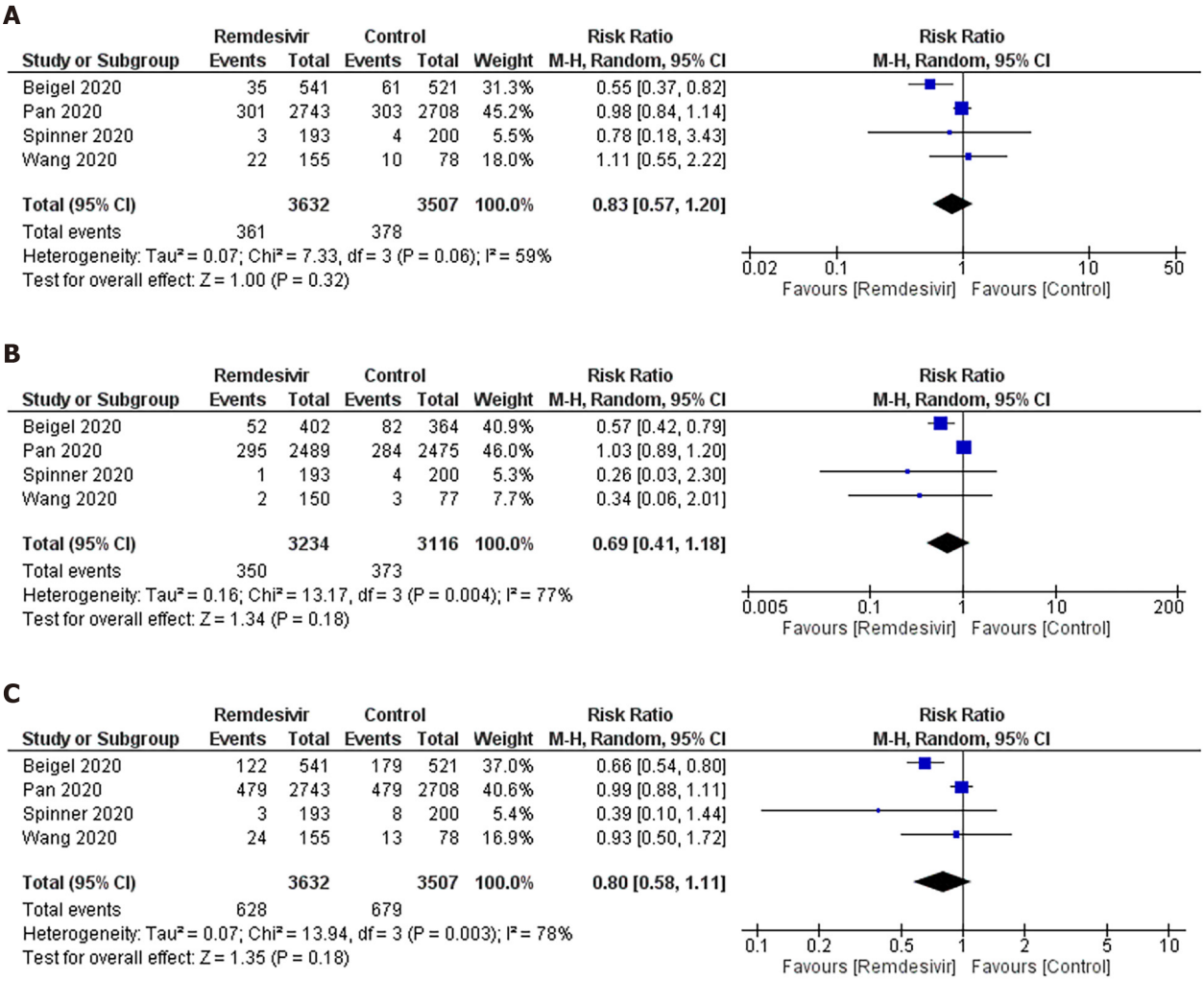


Figure 2 Meta-analytic summary through a random-effect model. A: Mortality data; B: Ventilation data; C: Composite mortality and ventilation data.

Clinical recovery: Three trials reported clinical recovery. Patients treated with remdesivir had higher rates of clinical recovery than those receiving placebo (RR: 1.10; 95%CI: 1.04–1.16; I^2 = 0%, Figure 3). On sensitivity analysis, the RR was 1.10 (95%CI: 1.02–1.18; I^2 = 0%). The NNT to cause one additional improvement in clinical recovery was 14.3 (95%CI: 9.1–33.3). GRADE approach evidence quality was moderate. The sensitivity analysis showed a similar trend in clinical recovery outcome.

Meta-analytic summary of safety outcomes

Adverse events: Three trials reported 520 adverse events in 880 participants of the remdesivir groups and 466 of 794 participants in the placebo-treated groups (Figure 4). There was no significant difference in the RR of adverse events between remdesivir and placebo-treated patients (RR: 1.05; 95%CI: 0.861.27; I^2 = 77%). The sensitivity analysis showed a similar trend in adverse event outcome. GRADE approach evidence quality was moderate.

Adverse events of grade 3 or 4: Three trials reported 315 grade 3 or 4 adverse events in 866 participants of the remdesivir groups and 340 of 780 participants in the placebo groups (Figure 5A). Patients receiving remdesivir were 11% less likely to experience grade 3 or 4 adverse events than placebo-treated patients (RR: 0.88; 95%CI: 0.79–0.99; I^2 = 0%). GRADE approach evidence quality was moderate. The NNT to cause one additional participant to experience fewer serious adverse events compared with placebo, was 20 (95%CI: 11.1–100). The sensitivity analysis did not suggest a reduced risk of adverse events in the remdesivir arm (RR: 0.87; 95%CI: 0.71–1.07; I^2 = 10%).

Serious adverse events: Three trials reported 186 serious adverse events in 880 participants of the remdesivir groups and 201 of 794 participants in the placebo-treated groups (Figure 5B). Participants were 25% less likely to experience serious adverse

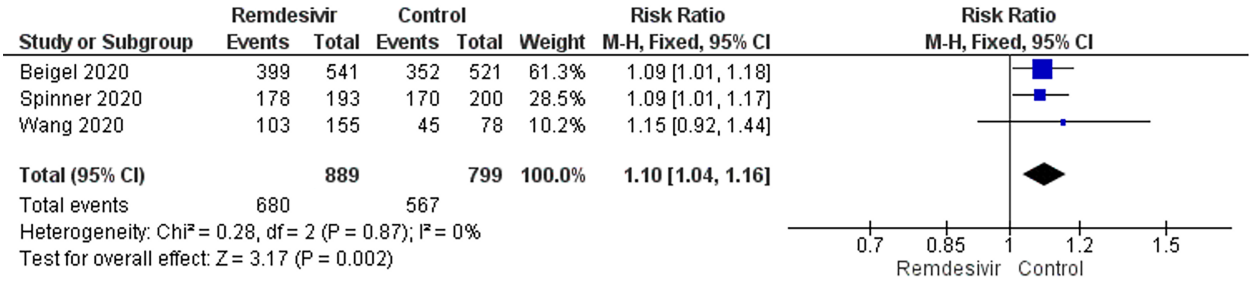


Figure 3 Meta-analytic summary of clinical recovery data through a fixed-effect model.

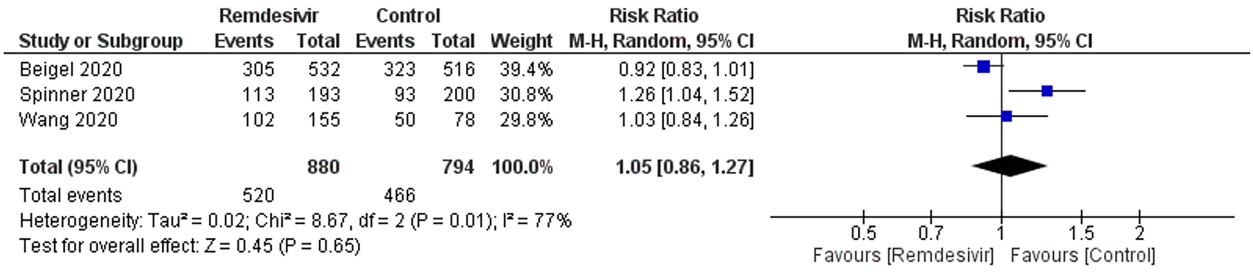


Figure 4 Meta-analytic summary of adverse events data through a random-effect model.

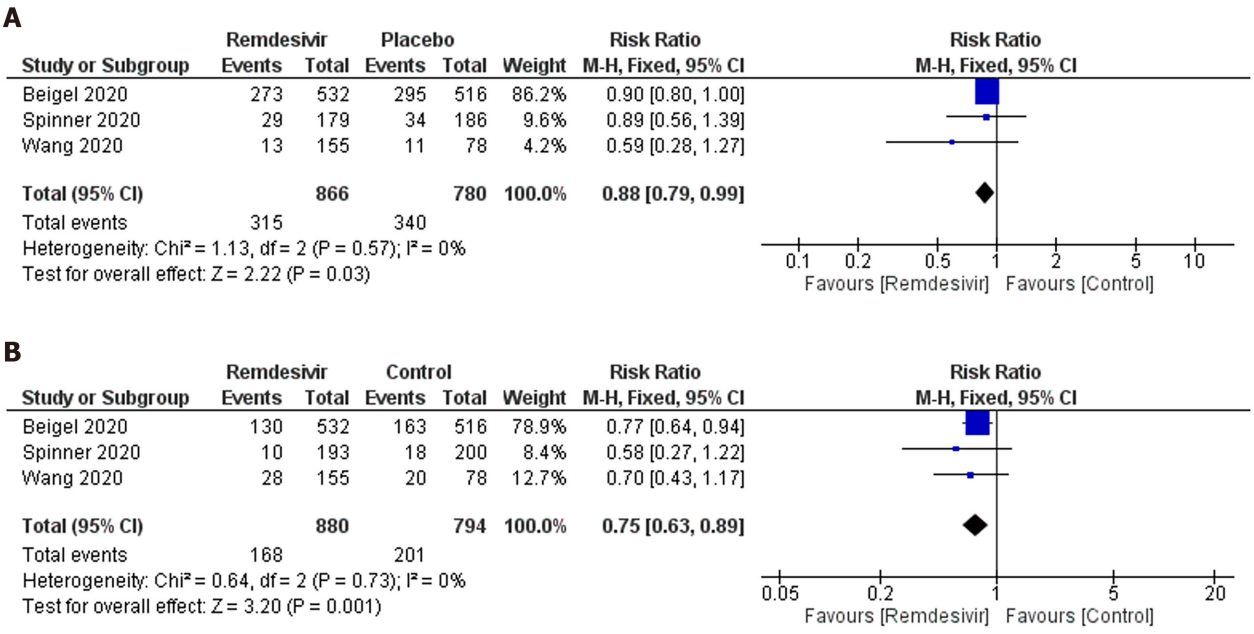


Figure 5 Meta-analytic summary through a fixed-effect model. A: Grade 3 or 4 adverse events data; B: Serious adverse event data.

events in the remdesivir group compared with the placebo-treated group (RR: 0.75; 95%CI: 0.63–0.89); I^2 = 0%; GRADE approach evidence quality was moderate). On sensitivity analysis, the RR was 0.76 (95%CI: 0.64–0.92; I^2 = 0%). The NNT to cause one additional participant to experience fewer serious adverse events compared with placebo, was 16.7 (95%CI: 11.1–50). The sensitivity analysis showed a similar trend in this outcome.

DISCUSSION

The findings of this pooled analysis suggest a trend of limited clinical benefits with the use of remdesivir in COVID-19 patients. More evidence based on double-blind

randomized controlled trials is required to evaluate its benefit in reducing mortality and the need of invasive ventilation in the severe COVID-19 patients.

Currently no effective antiviral drugs are available to treat COVID-19. Only dexamethasone showed a mortality benefit in severe COVID-19 patients requiring respiratory support[22]. During the initial period of the pandemic, remdesivir was used on a compassionate basis outside of randomized controlled trials in COVID-19 patients based on in vitro and animal studies. In May 2020, the Food and Drug Administration (FDA) granted the emergency use authorization to use remdesivir in severe COVID-19 patients. In October 2020, emergency use authorization was extended to all hospitalized patients[23]. Our meta-analysis suggests no major safety concerns with its use. Our findings support the emergency use authorization by the United States FDA in the absence of availability of better antiviral drugs. However, remdesivir alone does not seem to be a promising option. The discovery of better antiviral drug should go beyond it.

Included studies presented earlier findings of efficacy based on randomized controlled trials. Beigel *et al*[14] (the ACTT trial) reported a mortality benefit. Three other studies including the WHO solidarity trial observed no difference at the end of the study period[15,20,21]. In case of ventilatory support requirement, Beigel *et al*[14] suggested a beneficial effect. Two smaller trials observed a trend of reduced risk of ventilation[20,21], while the WHO solidarity trial did not find any difference[15]. A noncomparative study by Antinori *et al*[24], reported 22.2% of the patients on invasive ventilation showed improvement after remdesivir treatment. In case of clinical recovery, three trials reported trends of improvement[14,20,21]. Beigel *et al*[14] and Spinner *et al*[20] found that remdesivir was superior to placebo in shortening of clinical recovery time, whereas Wang *et al*[21] did not find any statistically significant clinical benefit with remdesivir in severe COVID-19 patients. Beigel *et al*[14] found that the median recovery time was shorter in patients treated with remdesivir than it was with placebo treatment (10 d *vs* 15 d), whereas in the study by Wang *et al*[21] the recovery time was 21 d in the remdesivir group compared with 23 d the placebo group. The WHO solidarity trial did not report clinical recovery with the study medications[15]. Moreover, there was a decreased duration of initial hospitalization (12 d *vs* 17 d) and a reduction in the number of days in which patients received oxygen (13 d *vs* 21 d) if they were on oxygen at baseline, in the remdesivir-treated group as compared to placebo. However, it did not reduce the reoccurrence of a need for oxygen[14]. Noncomparative studies by Antinori *et al*[24] and Grein *et al*[25] also observed that remdesivir can provide a greater benefit to patients with pneumonia requiring oxygen therapy or non-invasive ventilation than to those receiving mechanical ventilation. Thus, early intervention with remdesivir can provide increased clinical benefit with fewer adverse events[24]. None of the studies included in the present review focused on virological cure. Severe COVID-19 patients have high viral loads and generally need an longer duration to become negative than those with low viral loads[26,27]. A study by Antinori *et al*[24] found a 100% SARS-COV-2 negative conversion rate with remdesivir, and it occurred after a median period of 12 d (interquartile range 9.25–16.75) after starting the treatment.

Remdesivir has a better or comparable tolerability profile than placebo. The findings of safety analyses should be interpreted cautiously as rare and serious adverse events are usually identified with the widespread use of any drug outside of clinical trials[28,29]. Limited information is available for the use of remdesivir in pregnant women and patients with hepatic or renal impairment[30,31]. This is especially important from the aspect of high prevalence of acute kidney injury during and its associated mortality in hospitalized COVID-19 patients[32]. Abnormal liver function test values are common in COVID-19 patients[33,34].

Our study has several limitations. Although there was a meticulous search to identify published studies on PubMed and Google Scholar as well as preprint versions of studies on Medrxiv and other databases, we could identify only four published randomized trials to date. The findings of this study could indicate trends rather than confirmation due to non-consideration of the impact of co-morbidities and severity status. We could not analyze the impact of remdesivir on virological cure. While this study was under peer-review, few meta-analyses of remdesivir against COVID-19 have been published. The earlier studies included randomized and observational studies[35,36], did not perform sensitivity analyses[37-39] and did not include quality assessment of efficacy and safety parameters as per the GRADE approach[37-39].

CONCLUSION

The benefits of remdesivir over placebo were significant only in causing higher rates of clinical cure. Use of remdesivir can be continued on a compassionate basis in the absence of specific antiviral drugs. The evidence is based on only four clinical trials.

ARTICLE HIGHLIGHTS

Research background

There are no specific antiviral drugs currently available to combat coronavirus disease 2019 (COVID-19) caused by novel virus severe acute respiratory syndrome coronavirus 2. This has promoted the evaluation of various previously approved drugs as an effective treatment for COVID-19. Remdesivir is one such repurposed drug currently under investigation against COVID-19.

Research motivation

This study investigated whether remdesivir is an effective and safe option to treat COVID-19 patients.

Research objectives

In this study, the authors aimed to provide a meta-analytic summary of the efficacy and safety outcomes in COVID-19 patients with the use of remdesivir as compared with control interventions.

Research methods

A literature search was conducted to identify studies published through October 28, 2020. Randomized controlled trials of remdesivir plus any other interventions including placebo were included. The quality assessment of all included studies for methodological characteristics was performed with the revised Cochrane risk of bias assessment tool for randomized controlled clinical trials. The efficacy outcome variables were mortality, need for mechanical ventilation, composite mortality and ventilation and clinical recovery rate. The safety outcome variables were overall adverse events, grade 3 or 4 adverse events and the serious adverse events rate.

Research results

We included a total of four randomized controlled trials in this meta-analysis. Three studies were considered to have a low risk and one study was considered to have some concerns in the overall risk of bias assessment. Remdesivir- and placebo-treated patients did not differ in mortality, rate of mechanical ventilation and composite mortality and ventilation rate outcomes. A sensitivity analysis suggested a reduced risk of ventilation and composite mortality and ventilation with remdesivir on exclusion of open-label studies. Remdesivir-treated patients showed higher rates of clinical recovery than placebo-treated patients. Remdesivir and placebo-treated patients did not differ in the overall occurrence of adverse events. Remdesivir-treated patients were at lower risk of grade 3 or 4 adverse events and serious adverse events than placebo-treated patients. The GRADE approach suggested moderate quality of evidence for all efficacy and safety outcomes.

Research conclusions

The effect of remdesivir over placebo was not significant for mortality or the rate of ventilation. However, remdesivir may provide higher rates of clinical cure. There are no major safety concerns with the use of remdesivir. Its use can be continued on a compassionate basis in the absence of specific antiviral drugs.

Research perspectives

The current evidence is based on four clinical trials only. More evidence based on double-blind randomized controlled trials in different disease-severity populations is required to evaluate the benefits of remdesivir in COVID-19 patients.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 February 28; 9(1): 88-100

DOI: 10.13105/wjma.v9.i1.88

ISSN 2308-3840 (online)

META-ANALYSIS

Health-related quality of life in patients that have undergone liver resection: A systematic review and meta-analysis

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Supported by Grant-in-Aid from JSPS KAKENHI, No. JP 20K10404 (to Mizuguchi T); the Hokkaido Hepatitis B Litigation Orange Fund, No. 2059198; Terumo Life Science Foundation, No. 2000666;

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Abstract

BACKGROUND

Mortality after hepatectomy has decreased, and the quality of various surgical approaches to hepatectomy have been evaluated. Various assessments of quality of life (QOL) after hepatectomy have been developed and investigated in different clinical settings.

AIM

To conduct a systematic review and meta-analysis to examine two clinical topics: Laparoscopic hepatectomy *vs* open hepatectomy, and preoperative QOL status *vs* postoperative QOL status.

Pfizer Health Research Foundation, No. 2000777; Daiichi Sankyo Company, No. 2109540; Shionogi and Co., No. 2109493; MSD, No. 2099412; Takeda, No, 2000555; Sapporo Doto Hospital, No. 2039118; Noguchi Hospital, No. 2029083; Doki-kai Tomakomai Hospital, No. 2059203; and Tsuchida Hospital, No. 2069231.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): E

Received: January 22, 2021
Peer-review started: January 22, 2021
First decision: February 10, 2021
Revised: February 10, 2021
Accepted: February 25, 2021

METHODS

A systematic literature search was performed using PubMed and MEDLINE, including the Cochrane Library Central. The following inclusion criteria were set for inclusion in this meta-analysis: (1) Studies comparing preoperative QOL and postoperative QOL; and (2) Studies comparing QOL between laparoscopic hepatectomy and open hepatectomy.

RESULTS

A total of 8 articles were included in this meta-analysis. QOL was better after laparoscopic hepatectomy than after open hepatectomy.

CONCLUSION

The outcomes of evaluations of QOL after hepatectomy can depend on the type of questionnaire used, the timing of the assessment, and the etiology of the hepatic disease.

Key Words: Quality of life; Hepatectomy; Laparoscopy; Transarterial chemoembolization; Functional Assessment of Cancer Therapy–Hepatobiliary; 36-Item Short-Form Health Survey; European Organisation for Research and Treatment of Cancer Quality of Life Core Questionnaire

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Core Tip: A systematic review and meta-analysis of post-hepatectomy quality of life (QOL) assessments were conducted. A total of ten studies were included in the meta-analysis. QOL was better after hepatectomy than after transarterial chemoembolization. QOL was also better after laparoscopic hepatectomy than after open hepatectomy. The outcomes of post-hepatectomy QOL evaluations could depend on the type of questionnaire used, the timing of the assessment, and the etiology of the hepatic disease.

Citation: Ishinuki T, Ota S, Harada K, Tatsumi H, Harada K, Miyanishi K, Nagayama M, Takemasa I, Ohyanagi T, Hui TT, Mizuguchi T. Health-related quality of life in patients that have undergone liver resection: A systematic review and meta-analysis. *World J Meta-Anal* 2021; 9(1): 88-100
URL: https://www.wjgnet.com/2308-3840/full/v9/i1/88.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i1.88

INTRODUCTION

Recently, hepatectomy has become safe, and the mortality rate of the procedure is now less than 1%[1,2]. Besides surgery, various other approaches have been developed for managing liver tumors, such as ablation, chemotherapy, molecular targeted therapy, and immunotherapy[3,4]. Furthermore, various surgical approaches have been developed, such as laparoscopic hepatectomy, robot-assisted hepatectomy, hybrid methods, hand-assisted methods, and classic open hepatectomy[5-9]. Therefore, selecting the optimal approach is essential for ensuring patients receive high-quality treatment.

Patient-reported outcomes (PRO) are considered to be gold-standard methods for evaluating quality of life (QOL) and comparing different management strategies[10]. Various PRO, such as the Functional Assessment of Cancer Therapy–Hepatobiliary (FACT-Hep)[11], the 36-Item Short-Form Health Survey (SF-36)[12], the European Organisation for Research and Treatment of Cancer Quality of Life Core Questionnaire (QLQ-C30)[13], the EuroQol 5-dimension, 5-level questionnaire[14], and others, have been investigated in patients who underwent hepatectomy. The FACT-Hep consists of 5 subscales, physical well-being, social well-being, emotional well-being, functional well-being, and the hepatobiliary cancer subscale[15]. The sum of the scores for the five subscales gives a total score ranging from 0 to 180. A higher score indicates better QOL. The SF-36 consists of eight subscales, which are used to produce a physical

Article in press: February 25, 2021
Published online: February 28, 2021

P-Reviewer: Donadon M, Kordzaia D, Lo Tesoriere R
S-Editor: Wang JL
L-Editor: A
P-Editor: Li X

component score and a mental component score[16]. The EORTC developed the QLQ-C30. The QLQ-C30 consists of three subscales: global health status, functional scales, and symptom scales[17]. Each subscale gives a score ranging from 0 to 100. Higher scores in the global health status and functional scales represent better QOL.

Although many studies have investigated PRO-QOL after hepatectomy, even the best QOL questionnaires are imprecise. Also, the timing of the evaluation is usually unknown. We attempted to examine QOL in patients who had undergone hepatectomy. The first clinical question we investigated was whether postoperative QOL was better among patients who underwent hepatectomy or transarterial chemoembolization (TACE). The second question was whether QOL was better among patients who underwent laparoscopic hepatectomy or classic open hepatectomy. Finally, we compared the changes in QOL scores seen after hepatectomy. This systematic review and meta-analysis examined the current status of QOL studies of patients who underwent hepatectomy. In addition, it revealed a future clinical question and provided an idea for a future clinical study.

MATERIALS AND METHODS

Literature search

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement guidelines were followed when obtaining and reporting the meta-analysis data[18]. The PICOS scheme was followed when reporting the inclusion criteria. A systematic literature search was performed independently by two authors (Ishinuki T and Ota S) using PubMed and MEDLINE, including the Cochrane Library. The search was limited to human studies whose findings were reported in English. No restriction was set for the type of publication, the publication date, or publication status. Patients of any age or sex who underwent liver resection for any type of hepatic lesion were considered as outlined in the PICOS scheme. The search strategy was based on different combinations of words for each database. For the PubMed database the following combination was used: ("qol"[All Fields] AND ("liver"[MeSH Terms] OR "liver"[All Fields] OR "livers"[All Fields] OR "liver s"[All Fields]) AND ("surgery"[MeSH Subheading] OR "surgery"[All Fields] OR "surgical procedures, operative"[MeSH Terms] OR ("surgical"[All Fields] AND "procedures"[All Fields] AND "operative"[All Fields]) OR "operative surgical procedures"[All Fields] OR "general surgery"[MeSH Terms] OR ("general"[All Fields] AND "surgery"[All Fields]) OR "general surgery"[All Fields] OR "surgery s"[All Fields] OR "surgerys"[All Fields] OR "surgeries"[All Fields]). For the Medline database, the following combination was used: (QOL and Liver and Surgery).

Study selection

The two independent authors screened the titles and abstracts of the primary studies identified in the database search. Duplicate studies were excluded. The following inclusion criteria were set for inclusion in the meta-analysis: (1) Studies comparing preoperative QOL and postoperative QOL in patients who underwent liver resection for any type of hepatic lesion; (2) Studies comparing QOL between laparoscopic hepatectomy and open hepatectomy in patients who underwent liver resection for any type of hepatic lesion; (3) Studies reporting at least one QOL outcome; and (4) If the same institute reported more than one study, only the most recent or the highest level study was included.

The following exclusion criteria were set: (1) Original studies assessing the outcomes of liver transplantation; (2) Review articles, letters, comments, and case reports; and (3) Studies for which it was impossible to retrieve or calculate the data of interest. The Cohen kappa statistic was used to quantify the agreement between the investigators.

The protocol was registered with PROSPERO (#CRD42021225970).

Data extraction

The same two authors extracted the following primary data: (1) The questionnaires used for each QOL evaluation; (2) The first author, year of publication, and type of study; (3) The etiology of the disease and the number of times each intervention was performed; and (4) The timing of the evaluations. The reasons why studies were excluded from the full-text evaluation are shown in Supplementary Tables 1-3. All excluded references are listed in the supplemental references.

Risk of bias assessment

The Newcastle–Ottawa Scale (NOS) was used to assess the quality of the included studies, as they included observational studies (http://www.ohri.ca/). The NOS consists of three domains, patient selection, comparability of study groups, and outcome assessment. The minimum risk of bias gain 9 points. We considered studies that scored ≥ 7, 4-6, and < 4 to be high quality, moderate quality, and low quality, respectively[19].

Statistical analyses

All analyses were performed using the RevMan software (version 5.3.; The Cochrane Collaboration). The mean differences (MD) between groups were calculated for continuous variables. The interquartile ranges of the data were transformed by dividing them by 1.35 to produce alternative standard deviation values[20]. Multiple means and standard deviations were combined using the StatsToDo online web program (https://www.statstodo.com/index.php).

The χ^2 test was used to evaluate heterogeneity, and the Cochran Q and I^2 statistics were reported. The I^2 value describes the percentage variation between studies in degrees of freedom. Low, moderate, and high heterogeneity were defined based on cut-off values of 25%, 50%, and 75%, respectively, using the obtained I^2 test values[21].

All results were considered significant at P values of < 0.05.

RESULTS

Study selection

The literature search yielded 248 articles, and the abstracts were reviewed by two independent researchers (Figure 1). Of these, 30 articles were selected for full-text review. Two articles were excluded due to different comparison. Nine articles were excluded due to no data description being provided. Eleven articles were excluded as they did not involve appropriate timepoints. Detailed information about the excluded articles is shown in Supplementary Tables 1-3. Finally, a total of 8 articles were included in this meta-analysis (Table 1). Two studies used the FACT-Hep[22,23], four studies used the SF-36[24-27], and two studies used the QLQ-C30[28,29]. None of them were randomized controlled studies.

FACT-Hep

The FACT-Hep was used to compare QOL before and after hepatectomy. None of the FACT-Hep domains differed significantly from their preoperative levels at 3 mo (Figure 2A) or 12 mo (Figure 2B), although several domains at 3 mo after hepatectomy tends to be better than those at 12 mo.

SF-36

The SF-36 was used to compare QOL between laparoscopic hepatectomy and open hepatectomy at 3-6 mo after treatment (Figure 3A). Although the physical component score did not differ significantly between the groups (P = 0.08), the mental component score for the laparoscopic hepatectomy group was significantly more favorable than that for the open hepatectomy group (P = 0.001). On the other hand, the physical component scores and mental component scores seen at 3 mo after hepatectomy were significantly more favorable than those observed before hepatectomy (Figure 3B; P = 0.04 and P = 0.02, respectively).

QLQ-C30

The QLQ-C30 was used to evaluate QOL at 6 mo and 12 mo after hepatectomy (Figure 4). No significant differences in global health, emotional function, or social function were observed between the preoperative assessment and 6 mo or 12 mo after hepatectomy. However, the patients' preoperative physical function scores were better than those seen at 6 mo or 12 mo after hepatectomy (P = 0.0004 and P = 0.04, respectively). Although role function and cognitive function differed significantly between the preoperative assessment and 6 mo after hepatectomy (P = 0.01 and P = 0.02, respectively), they did not differ significantly between the preoperative assessment and 12 mo after hepatectomy.

Table 1 Data extracted from the included studies

Questionnaire	Ref.	Type of study	Etiology	Number of each intervention	Timing of the evaluations
FACT-Hep	Martin *et al*[22], 2007	Prospective	HCC; CCC; CRLM	24 hepatectomies	Pre, 6 wk, 3 mo
	Liu *et al*[23], 2012	Retrospective	HCC	65 hepatectomies; 50 chemotherapies	Pre, 3 mo, 6 mo, 9 mo, 12 mo
SF-36	Giuliani *et al*[24], 2014	Retrospective	Miscellaneous	38 open hepatectomies; 28 laparoscopic hepatectomies	6 mo, 12 mo
	Qiu *et al*[25], 2015	Prospective	Hemangioma	344 enucleations; 386 hepatectomies	Pre, 1 mo, 3 mo, 6 mo
	Chiu *et al*[26], 2018	Prospective	HCC	332–324 hepatectomies	Pre, 3 mo, 6 M mo
	Liu *et al*[27], 2019	Prospective	Hemangioma	73 open hepatectomies; 73 laparoscopic hepatectomies	Pre, 1 mo, 3 mo
QLQ-C30	Dasgupta *et al*[28], 2008	Prospective	CRLM; CCC; HCC	101–33 hepatectomies	Pre, 6 mo, 12 mo, 36–48 mo
	Rees *et al*[29], 2012	Prospective	CRLM	232–193 hepatectomies	Pre, 3 mo, 6 mo, 12 mo

QOL: Quality of life, HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; CCC: Cholangiocellular carcinoma; CRLM: Colorectal liver metastasis; FACT-Hep: Functional Assessment of Cancer Therapy–Hepatobiliary; SF-36: The 36-Item Short-Form Health Survey; QLQ-C30: The European Organisation for Research and Treatment of Cancer Quality of Life Core Questionnaire.

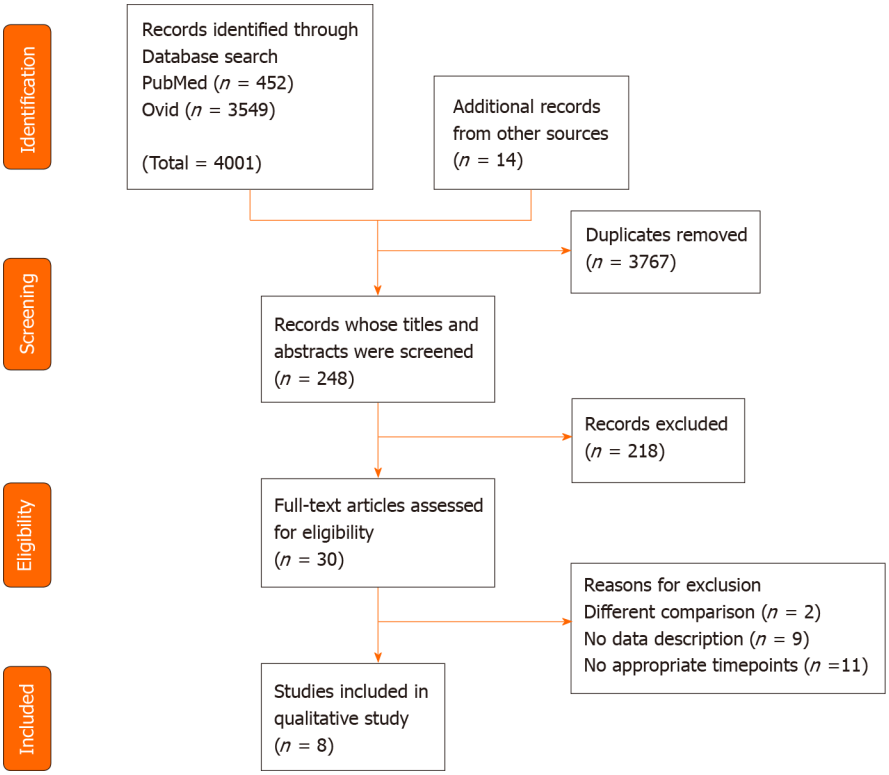


Figure 1 Flow diagram of systematic reviews and meta-analyses.

Quality assessment

The quality assessment was conducted using the NOS score (Supplementary Table 4). Three studies were of moderate quality, and seven studies were of high quality.

DISCUSSION

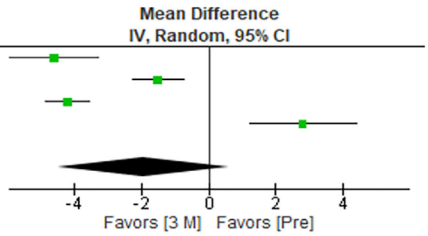
Liver resection has become a safe surgical procedure for liver tumors and is now used

A

Physical well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	3 M Mean	3 M SD	3 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	22	2.9	20	26.6	1.4	66	24.6%	-4.60 [-5.92, -3.28]	2001
Martin 2007	3	1.4	24	4.5	1.3	24	25.7%	-1.50 [-2.26, -0.74]	2007
Liu 2012	19	1.5	65	23.2	2.1	65	25.9%	-4.20 [-4.83, -3.57]	2012
Toro 2012	23.9	2.1	14	21.1	2.2	14	23.8%	2.80 [1.21, 4.39]	2012
Total (95% CI)			**123**			**169**	**100.0%**	**-1.94 [-4.48, 0.61]**	

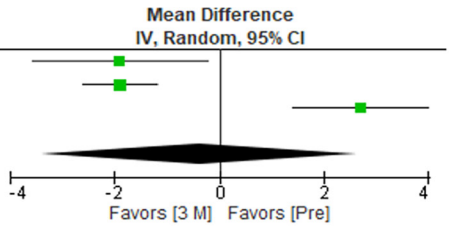
Heterogeneity: Tau² = 6.39; Chi² = 84.88, df = 3 (P < 0.00001); I² = 96%
Test for overall effect: Z = 1.49 (P = 0.14)



Social well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	3 M Mean	3 M SD	3 M Total	Weight	Mean Difference IV, Random, 95% CI
Poon 2001	20.2	3.5	20	22.1	2.9	66	31.9%	-1.90 [-3.59, -0.21]
Liu 2012	17.2	1.9	65	19.1	2.2	65	34.8%	-1.90 [-2.61, -1.19]
Toro 2012	22.3	1.7	14	19.6	1.8	14	33.3%	2.70 [1.40, 4.00]
Total (95% CI)			**99**			**145**	**100.0%**	**-0.37 [-3.38, 2.65]**

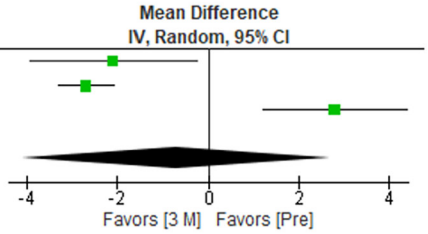
Heterogeneity: Tau² = 6.67; Chi² = 38.58, df = 2 (P < 0.00001); I² = 95%
Test for overall effect: Z = 0.24 (P = 0.81)



Emotional well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	3 M Mean	3 M SD	3 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	14.4	4	20	16.5	2.4	66	32.1%	-2.10 [-3.95, -0.25]	2001
Liu 2012	12.4	2	65	15.1	1.6	65	35.0%	-2.70 [-3.32, -2.08]	2012
Toro 2012	22.7	2.1	14	19.9	2.2	14	32.9%	2.80 [1.21, 4.39]	2012
Total (95% CI)			**99**			**145**	**100.0%**	**-0.70 [-4.10, 2.70]**	

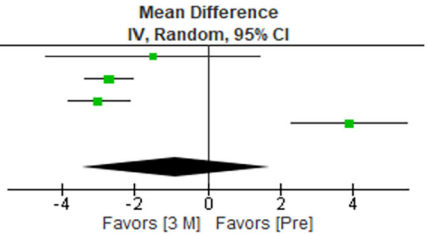
Heterogeneity: Tau² = 8.50; Chi² = 39.73, df = 2 (P < 0.00001); I² = 95%
Test for overall effect: Z = 0.40 (P = 0.69)



Functional well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	3 M Mean	3 M SD	3 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	20.2	6.3	20	21.7	4.3	66	20.5%	-1.50 [-4.45, 1.45]	2001
Martin 2007	19.5	1.3	24	22.2	1	24	27.3%	-2.70 [-3.36, -2.04]	2007
Liu 2012	18.2	2.8	65	21.2	2.1	65	27.0%	-3.00 [-3.85, -2.15]	2012
Toro 2012	21.9	2	14	18	2.3	14	25.2%	3.90 [2.30, 5.50]	2012
Total (95% CI)			**123**			**169**	**100.0%**	**-0.87 [-3.47, 1.72]**	

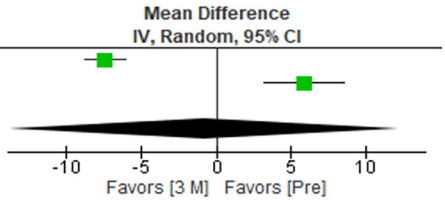
Heterogeneity: Tau² = 6.29; Chi² = 61.88, df = 3 (P < 0.00001); I² = 95%
Test for overall effect: Z = 0.66 (P = 0.51)



Hepatobiliary cancer subscale

Study or Subgroup	Pre Mean	Pre SD	Pre Total	3 M Mean	3 M SD	3 M Total	Weight	Mean Difference IV, Random, 95% CI
Liu 2012	50.1	3.8	65	57.5	4.3	65	50.4%	-7.40 [-8.80, -6.00]
Toro 2012	61.1	3.5	14	55.2	3.7	14	49.6%	5.90 [3.23, 8.57]
Total (95% CI)			**79**			**79**	**100.0%**	**-0.80 [-13.83, 12.23]**

Heterogeneity: Tau² = 87.27; Chi² = 74.97, df = 1 (P < 0.00001); I² = 99%
Test for overall effect: Z = 0.12 (P = 0.90)

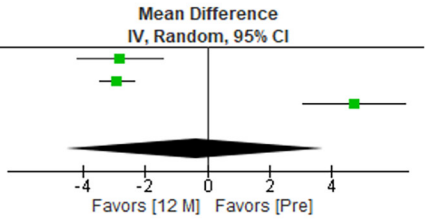


B

Physical well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	12 M Mean	12 M SD	12 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	22	2.9	20	24.8	0.9	10	33.1%	-2.80 [-4.19, -1.41]	2001
Liu 2012	19	1.5	65	21.9	1.8	65	34.2%	-2.90 [-3.47, -2.33]	2012
Toro 2012	23.9	2.1	14	19.2	2.3	14	32.7%	4.70 [3.07, 6.33]	2012
Total (95% CI)			**99**			**89**	**100.0%**	**-0.38 [-4.50, 3.74]**	

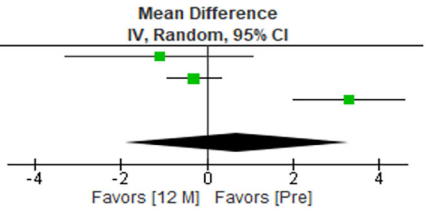
Heterogeneity: Tau² = 12.84; Chi² = 75.22, df = 2 (P < 0.00001); I² = 97%
Test for overall effect: Z = 0.18 (P = 0.85)



Social well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	12 M Mean	12 M SD	12 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	20.2	3.5	20	21.3	2.5	10	29.5%	-1.10 [-3.28, 1.08]	2001
Liu 2012	17.2	1.9	65	17.5	1.8	65	36.4%	-0.30 [-0.94, 0.34]	2012
Toro 2012	22.3	1.7	14	19	1.8	14	34.1%	3.30 [2.00, 4.60]	2012
Total (95% CI)			**99**			**89**	**100.0%**	**0.69 [-1.92, 3.30]**	

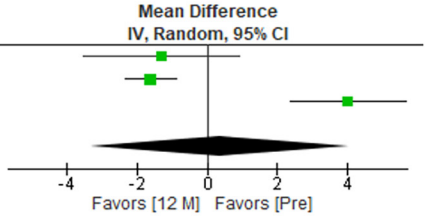
Heterogeneity: Tau² = 4.75; Chi² = 25.55, df = 2 (P < 0.00001); I² = 92%
Test for overall effect: Z = 0.52 (P = 0.60)



Emotional well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	12 M Mean	12 M SD	12 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	14.4	4	20	15.7	2.2	10	31.5%	-1.30 [-3.52, 0.92]	2001
Liu 2012	12.4	2	65	14	2.2	65	35.2%	-1.60 [-2.32, -0.88]	2012
Toro 2012	22.7	2.1	14	18.7	2.3	14	33.3%	4.00 [2.37, 5.63]	2012
Total (95% CI)			**99**			**89**	**100.0%**	**0.36 [-3.32, 4.04]**	

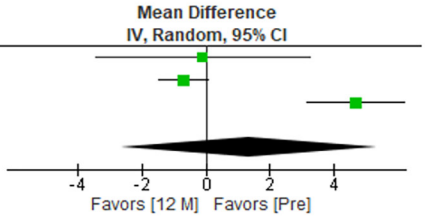
Heterogeneity: Tau² = 9.90; Chi² = 38.11, df = 2 (P < 0.00001); I² = 95%
Test for overall effect: Z = 0.19 (P = 0.85)



Functional well-being

Study or Subgroup	Pre Mean	Pre SD	Pre Total	12 M Mean	12 M SD	12 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Poon 2001	20.2	6.3	20	20.3	3.1	10	29.1%	-0.10 [-3.46, 3.26]	2001
Liu 2012	18.2	2.8	65	18.9	1.6	65	36.1%	-0.70 [-1.48, 0.08]	2012
Toro 2012	21.9	2	14	17.2	2.1	14	34.8%	4.70 [3.18, 6.22]	2012
Total (95% CI)			**99**			**89**	**100.0%**	**1.35 [-2.67, 5.37]**	

Heterogeneity: Tau² = 11.48; Chi² = 38.43, df = 2 (P < 0.00001); I² = 95%
Test for overall effect: Z = 0.66 (P = 0.51)



Hepatobiliary cancer subscale

Study or Subgroup	Pre Mean	Pre SD	Pre Total	12 M Mean	12 M SD	12 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Liu 2012	50.1	3.8	65	52.2	3.6	65	50.5%	-2.10 [-3.37, -0.83]	2012
Toro 2012	61.1	3.5	14	50.7	3.7	14	49.5%	10.40 [7.73, 13.07]	2012
Total (95% CI)			**79**			**79**	**100.0%**	**4.09 [-8.16, 16.34]**	

Heterogeneity: Tau² = 76.99; Chi² = 68.70, df = 1 (P < 0.00001); I² = 99%
Test for overall effect: Z = 0.65 (P = 0.51)

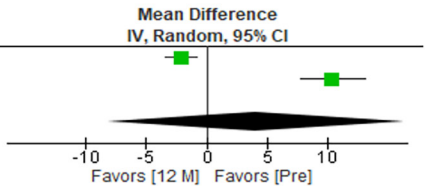


Figure 2 Functional Assessment of Cancer Therapy–Hepatobiliary scores before and at 3 mo, 12 mo after hepatectomy. A: Before and at 3 mo after hepatectomy; B: Before and at 12 mo after hepatectomy.

for living transplantation[2,3,5,13]. The clinical outcomes of hepatectomy have been reported based on quality assessments since 2000, and evidence has accumulated rapidly within the last decade[30]. We evaluated two crucial clinical questions in this study. The first was whether hepatectomy or TACE resulted in better QOL. The second was whether laparoscopic or open hepatectomy resulted in better QOL. Furthermore, we examined the changes in QOL seen at 3 mo, 6 mo, and 12 mo after hepatectomy.

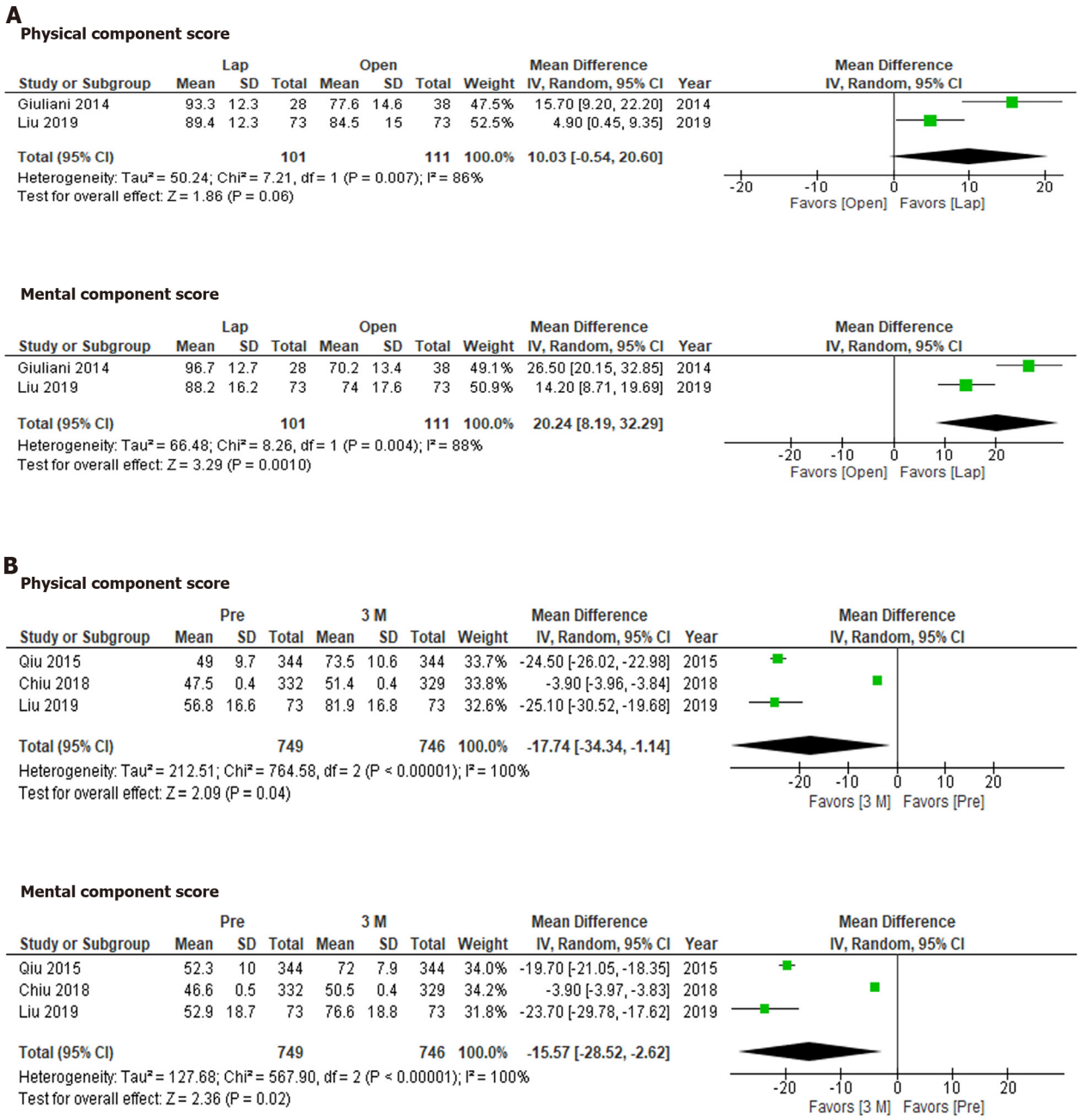


Figure 3 The 36-Item Short-Form Health Survey scores at 3 mo and 6 mo after laparoscopic or open hepatectomy or at 3 mo after hepatectomy. A: 3 mo and 6 mo after laparoscopic or open hepatectomy; B: 3 mo after hepatectomy.

The findings of postoperative QOL assessments can vary according to the type of questionnaire used, the surgical approach, the etiology of the disease, and the timing of the evaluations[30].

Most of the studies examined in the present review were conducted using a paper-based approach or face-to-face interviews. A more mobile approach would allow the comprehensive collection of a greater variety of data[31]. However, some questionnaires are not suitable for extensive prospective surveys due to cost issues. In addition, language translation is also an obstacle to international comparisons among questionnaires. Therefore, the statistical power of the studies was limited.

The FACT-Hep did not identify any significant changes in QOL after hepatectomy. Our results indicate that HCC patients' QOL recovered within 12 mo after hepatectomy. Although the QOL scores for each subdomain at 3 mo did not differ significantly from those observed before hepatectomy, the integrated mean tended to be more favorable at 3 mo after hepatectomy than before hepatectomy. This would depend on the condition of the patients who were eligible for the studies. Therefore, the QOL scores for these patients would have improved after hepatectomy.

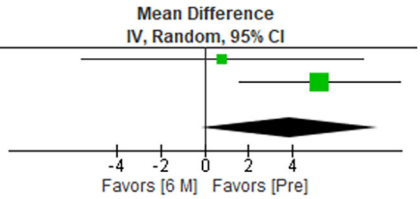
According to the SF-36, QOL was significantly better at 3 mo after hepatectomy than

A

Global health

Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	67.9	23.4	101	67.1	21.2	87	31.6%	0.80 [-5.58, 7.18]	2008
Ree 2012	76.3	19	232	71.1	20	211	68.4%	5.20 [1.56, 8.84]	2012
Total (95% CI)			**333**			**298**	**100.0%**	**3.81 [-0.20, 7.82]**	

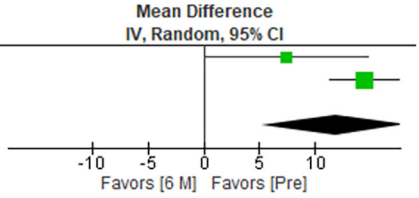
Heterogeneity: Tau² = 2.66; Chi² = 1.38, df = 1 (P = 0.24); I² = 27%
Test for overall effect: Z = 1.86 (P = 0.06)



Physical function

Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	74.4	24.4	97	66.9	25	80	38.0%	7.50 [0.18, 14.82]	2008
Ree 2012	98	15.9	232	83.6	17.4	211	62.0%	14.40 [11.29, 17.51]	2012
Total (95% CI)			**329**			**291**	**100.0%**	**11.78 [5.21, 18.34]**	

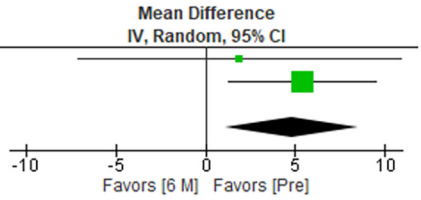
Heterogeneity: Tau² = 15.57; Chi² = 2.89, df = 1 (P = 0.09); I² = 65%
Test for overall effect: Z = 3.52 (P = 0.0004)



Role function

Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	73.2	32.4	102	71.3	30.6	87	17.1%	1.90 [-7.09, 10.89]	2008
Ree 2012	80.7	27.2	232	75.3	15.6	211	82.9%	5.40 [1.32, 9.48]	2012
Total (95% CI)			**334**			**298**	**100.0%**	**4.80 [1.08, 8.52]**	

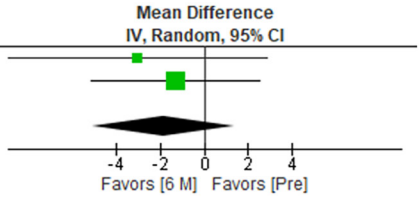
Heterogeneity: Tau² = 0.00; Chi² = 0.48, df = 1 (P = 0.49); I² = 0%
Test for overall effect: Z = 2.53 (P = 0.01)



Emotional function

Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	77.2	20.3	101	80.2	20.2	87	30.2%	-3.00 [-8.80, 2.80]	2008
Ree 2012	79.8	19.8	232	81.1	21.1	211	69.8%	-1.30 [-5.12, 2.52]	2012
Total (95% CI)			**333**			**298**	**100.0%**	**-1.81 [-5.01, 1.38]**	

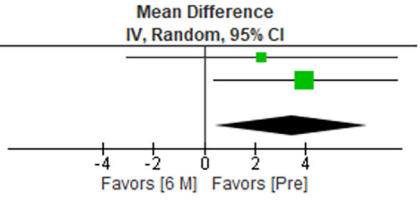
Heterogeneity: Tau² = 0.00; Chi² = 0.23, df = 1 (P = 0.63); I² = 0%
Test for overall effect: Z = 1.11 (P = 0.27)



Cognitive function

Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	84.5	17.5	101	82.2	19.8	87	31.4%	2.30 [-3.08, 7.68]	2008
Ree 2012	87.2	17.1	232	83.2	21.5	211	68.6%	4.00 [0.36, 7.64]	2012
Total (95% CI)			**333**			**298**	**100.0%**	**3.47 [0.45, 6.48]**	

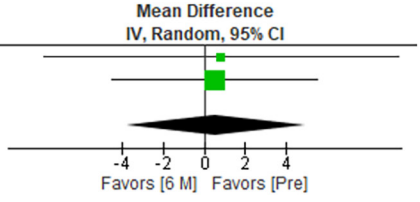
Heterogeneity: Tau² = 0.00; Chi² = 0.26, df = 1 (P = 0.61); I² = 0%
Test for overall effect: Z = 2.25 (P = 0.02)



Social function

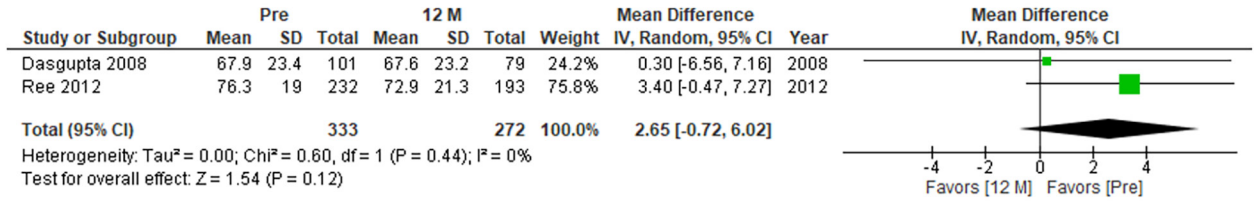
Study or Subgroup	Pre Mean	Pre SD	Pre Total	6 M Mean	6 M SD	6 M Total	Weight	Mean Difference IV, Random, 95% CI	Year
Dasgupta 2008	71.3	30.1	101	70.5	30.5	87	25.1%	0.80 [-7.89, 9.49]	2008
Ree 2012	78.5	26.8	232	78	27.1	211	74.9%	0.50 [-4.53, 5.53]	2012
Total (95% CI)			**333**			**298**	**100.0%**	**0.58 [-3.78, 4.93]**	

Heterogeneity: Tau² = 0.00; Chi² = 0.00, df = 1 (P = 0.95); I² = 0%
Test for overall effect: Z = 0.26 (P = 0.80)

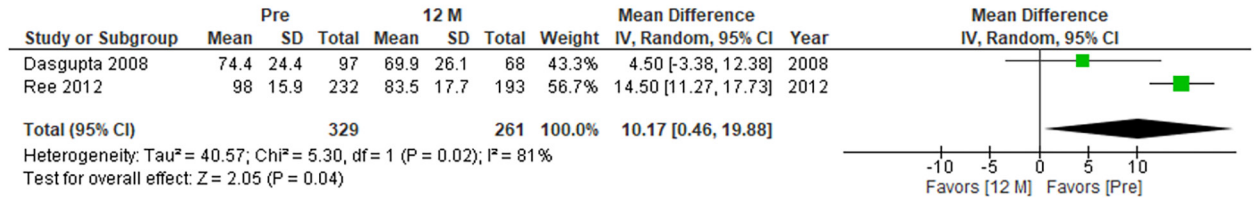


B

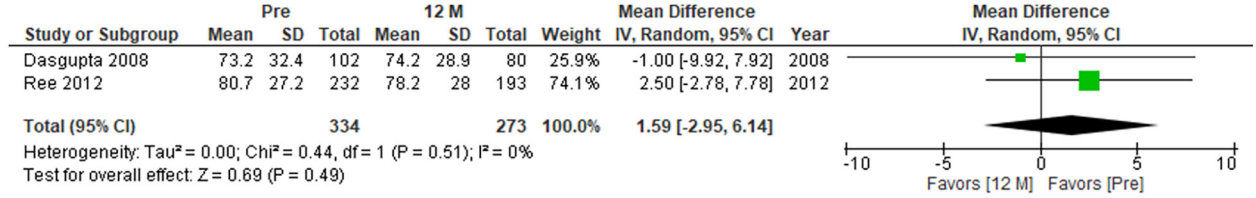
Global health



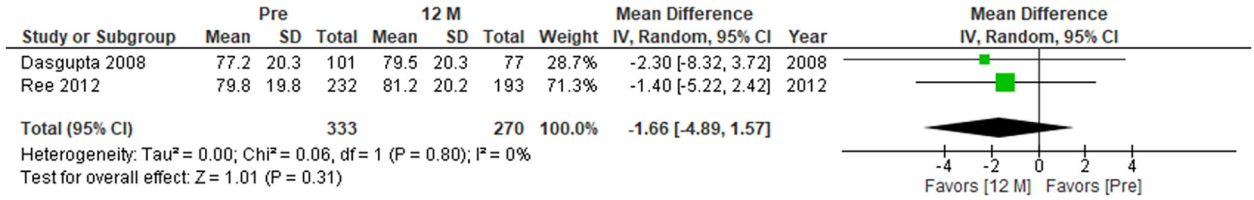
Physical function



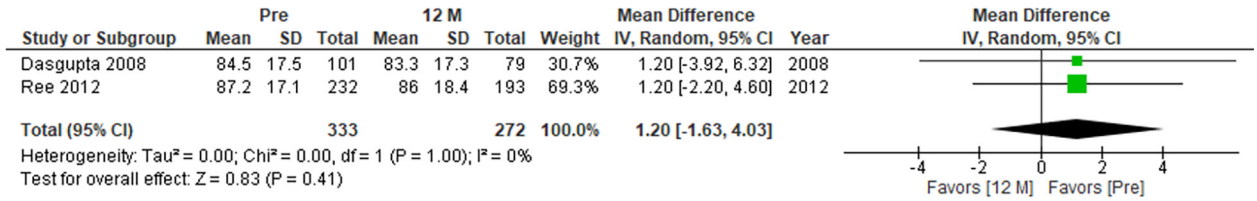
Role function



Emotional function



Cognitive function



Social function

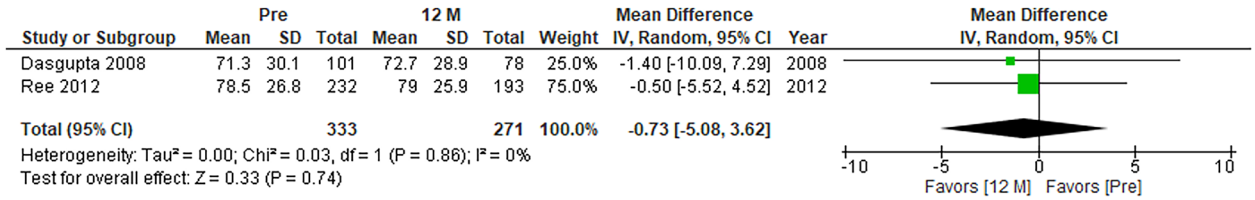


Figure 4 The European Organisation for Research and Treatment of Cancer Quality of Life Core Questionnaire scores at 6 mo and 12 mo after hepatectomy. A: 6 mo after hepatectomy; B: 12 mo after hepatectomy.

before hepatectomy. On the other hand, different results might have been obtained if the studies had involved asymptomatic patients[26]. Another concern is the sensitivity of each questionnaire. It is possible that the SF-36 is more sensitive than the FACT-Hep in these circumstances.

Laparoscopic hepatectomy has become the standard approach for liver resection[5,7,9]. It is considered that the reduced invasiveness associated with the minimal wound length of the laparoscopic approach allows patients to recover faster than is possible with the open approach[5]. As we demonstrated in this study, QOL could be better after laparoscopic hepatectomy than after open hepatectomy. In addition, the mental component scores of the patients that underwent laparoscopic hepatectomy were significantly better than their physical component scores. The reduced invasiveness of laparoscopic hepatectomy is considered to improve physical outcomes. However, this was not proven, presumably due to the long period of time between the surgery and the assessments. The degree to which mental QOL was preserved is a unique feature of laparoscopic hepatectomy.

The QLQ-C30 produced different results from the other QOL questionnaires. Physical function and role function had deteriorated significantly at 6 mo after hepatectomy, but had recovered at 12 mo after hepatectomy. The changes in the QLQ-C30 seen after hepatectomy seem to be more reasonable. They indicate that surgery itself temporarily reduces patients' QOL. In addition, the examined studies included asymptomatic patients who had metastatic liver tumors from colorectal cancer[28,29], which could also explain why QOL deteriorated after surgery.

CONCLUSION

Laparoscopic hepatectomy resulted in better QOL than open hepatectomy. The results of QOL evaluations performed after hepatectomy could depend on the type of questionnaire used, the timing of the assessment, and the etiology of the hepatic disease.

ARTICLE HIGHLIGHTS

Research background

The quality of life (QOL) assessment after hepatectomy has never been summarized. Therefore, comprehensive systematic review and meta-analysis would have great scientific value.

Research motivation

Lack of randomized controlled trial motivate us to plan prospective study. However, sample size calculation is difficult due to lack of the QOL value. This analysis would be helpful to conduct future trials.

Research objectives

Research objectives were to elucidate QOL after hepatectomy.

Research methods

Systematic review and meta-analysis was conducted according to PROSPERO guidelines with Preferred Reporting Items for Systematic Reviews and Meta-Analyses check lists.

Research results

A total of 8 articles were included in this meta-analysis. QOL was better after laparoscopic hepatectomy than after open hepatectomy. Physical and mental component score of the 36-Item Short-Form Health Survey at 3 mo was significantly better than before hepatectomy.

Research conclusions

The outcomes of evaluations of QOL after hepatectomy can depend on the type of questionnaire used, the timing of the assessment, and the etiology of the hepatic disease.

Research perspectives

The values from this study could be useful to plan future randomized control trial.

ACKNOWLEDGEMENTS

We thank Tan S and Nara M for their help in preparing this manuscript and valuable discussions.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
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ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 April 28; 9(2): 101-219



Published by Baishideng Publishing Group Inc

World Journal of Meta-Analysis

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World Journal of Meta-Analysis

Bimonthly Volume 9 Number 2 April 28, 2021

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The primary aim of *World Journal of Meta-Analysis* (*WJMA*, *World J Meta-Anal*) is to provide scholars and readers from various fields of clinical medicine with a platform to publish high-quality meta-analysis and systematic review articles and communicate their research findings online.

WJMA mainly publishes articles reporting research results and findings obtained through meta-analysis and systematic review in a wide range of areas, including medicine, pharmacy, preventive medicine, stomatology, nursing, medical imaging, and laboratory medicine.

INDEXING/ABSTRACTING

The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Saurabh Chandan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2308-3840/editorialboard.htm

PUBLICATION DATE
April 28, 2021

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E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Meta-Analysis

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World J Meta-Anal 2021 April 28; 9(2): 101-107

DOI: 10.13105/wjma.v9.i2.101

ISSN 2308-3840 (online)

OPINION REVIEW

Effect of resistance exercise on insulin sensitivity of skeletal muscle

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Author contributions: Zhao YC conceived the idea for the manuscript; Luo X, Li RR, and Li YN contributed to the writing of the manuscript; Wang B reviewed the literature and drafted the manuscript.

Supported by National Natural Science Foundation of China Youth Project, No. 31702024; Shandong Province Higher Educational Science and Technology Plan Project, No. J17KA258.

Conflict-of-interest statement: None.

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Abstract

Insulin resistance (IR) is the common pathophysiological basis of many metabolic diseases. IR is characterized by decreased glucose uptake in skeletal muscle and adipose tissue, especially in skeletal muscle. Skeletal muscle is the main target tissue of glucose uptake under insulin stimulation. Glucose uptake by skeletal muscle is complex, and it is controlled by many pathways. The PI3K/AKt/GSK-1 signaling pathway is not only the main pathway for insulin signal transduction but also an important mechanism for regulating blood glucose. From the binding of insulin to its receptors on the surface of target cells to the transportation of glucose from extracellular fluid to skeletal muscle, a series of signal transduction processes is completed, any of which potentially affects the physiological effects of insulin and leads to IR. Resistance exercise (RT) can reduce skeletal muscle IR and effectively improve blood glucose control and glycosylated hemoglobin level in patients with type 2 diabetes mellitus (T2DM). However, the exact mechanism by which RT improves skeletal muscle IR remains unclear. Therefore, this paper discusses the above problems by tracking the progress of the literature to deepen the correlation between RT and skeletal muscle insulin sensitivity and provide further evidence for the application of exercise therapy in IR. In conclusion, RT mainly improves insulin sensitivity of skeletal muscle by increasing muscle mass, microvascular blood flow, and glucose transporter-4 expression in skeletal muscle, as well as by reducing lipid accumulation and inflammation in skeletal muscle. Thus, it is potentially useful in the prevention and treatment of T2DM.

Key Words: Resistance exercise; Insulin sensitivity; Insulin resistance; Skeletal muscle; Microvascular blood flow; Muscle mass

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Manuscript source: Invited manuscript

Specialty type: Endocrinology and metabolism

Country/Territory of origin: China

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: February 25, 2021
Revised: March 10, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Wang CR
S-Editor: Wang JL
L-Editor: Wang TQ
P-Editor: Li JH



Core Tip: Both muscle contraction and hypoxia can mobilize the transfer of glucose transporter-4. Resistance exercises (RTs) can significantly increase limb muscle content and body fat content and significantly reduce blood glucose and glycosylated hemoglobin levels in patients with type 2 diabetes mellitus (T2DM). Lipid accumulation in skeletal muscle cells potentially leads to insulin resistance (IR). RT or acute RT can lower intracellular lipid accumulation in muscle, which helps reduce peripheral IR. It is suggested that the RT-induced microvascular blood flow increase helps improve glucose metabolism in patients with skeletal muscle IR and T2DM.

Citation: Wang B, Luo X, Li RR, Li YN, Zhao YC. Effect of resistance exercise on insulin sensitivity of skeletal muscle. *World J Meta-Anal* 2021; 9(2): 101-107
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/101.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.101

INTRODUCTION

Insulin resistance (IR) is the common pathophysiological basis of many metabolic diseases, and it is a consequence of decreased sensitivity of peripheral tissue to insulin, resulting in a higher demand for insulin to maintain normal metabolic homeostasis. IR is characterized by decreased glucose uptake in skeletal muscle and adipose tissue[1], especially in skeletal muscle[2]. Skeletal muscle is the main target tissue of glucose uptake under insulin stimulation. Eighty to ninety percent of insulin-stimulated glucose uptake occurs in skeletal muscle[3]. Glucose uptake by skeletal muscle is complex, and it is controlled by many pathways. The PI3K/AKt/GSK-1 signaling pathway is not only the main pathway for insulin signal transduction[4], but also an important mechanism for regulating blood glucose. From the binding of insulin to its receptors on the surface of target cells to the transportation of glucose from extracellular fluid to skeletal muscle, a series of signal transduction processes is completed, any of which potentially affects the biological effects of insulin and leads to IR (Figure 1). Genetic susceptibility, lack of physical activity, and obesity all lead to IR[5].

Resistance exercise (RT) is movement in which muscles overcome external resistance to actively contract and relax, which deviates from traditional aerobic exercise in that it exerts external resistance and emphasizes the exercise of muscle strength and endurance. RT can reduce skeletal muscle IR[6] and effectively improve blood glucose control and glycosylated hemoglobin (HbA1c) levels in patients with type 2 diabetes mellitus (T2DM)[7]. However, the exact mechanism by which RT improves skeletal muscle IR remains unclear. Therefore, this paper discusses the above problems by tracking the progress of the literature, locally and abroad, in order to deepen the correlation between RT and skeletal muscle insulin sensitivity and provide further evidence for the application of exercise therapy in IR.

RT INCREASES THE EXPRESSION OF GLUCOSE TRANSPORTER-4 IN SKELETAL MUSCLE

One of the most mature mechanisms of IR is the decreased expression of the glucose transporter-4 (GLUT4) protein in muscle, which may be related to mitochondrial dysfunction and decreased biosynthesis[8]. Sharma *et al*[9] discovered that the normal phosphorylation of AS160 serine-588 and threonine-642 in the PI3K pathway was significantly decreased in elderly patients with T2DM, and these two sites were considered to have an important effect on the translocation of GLUT4. The decrease or even interruption of the transfer of GLUT4 self-storage vesicles to the surface of muscle cells may be another important reason for the damage caused to the insulin signal pathway. As a single stimulating factor, exercise can promote the translocation of GLUT-4 to the cell membrane and enhance glucose uptake[10]. Both muscle contraction and hypoxia can mobilize the transfer of GLUT4. The energy supply characteristic of RT is that muscle contraction can also cause a hypoxic environment inside the muscle cells, whereas aerobic exercise cannot. According to the degree of IR

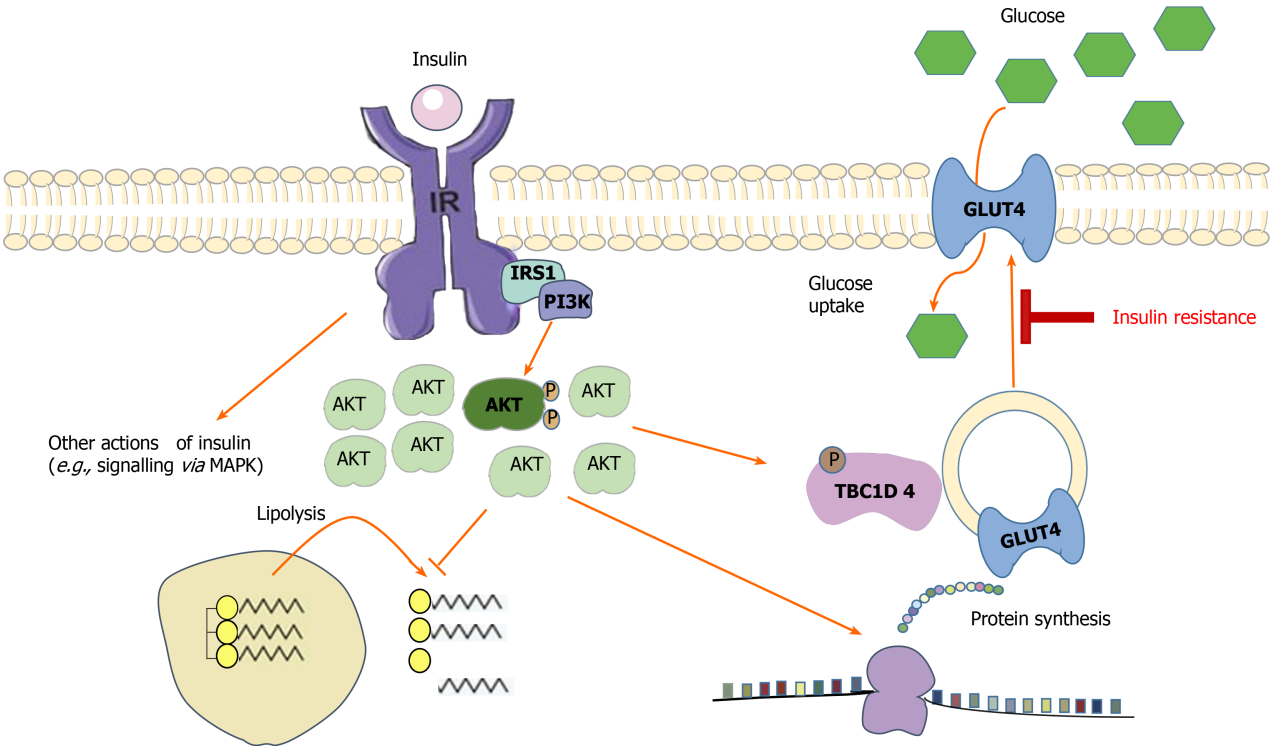


Figure 1 Mechanism of insulin resistance. IR: Insulin resistance; GLUT4: Glucose transporter-4.

in T2DM patients, adding the corresponding RT content in the exercise prescription can achieve a better intervention outcome.

The content of GLUT4 in the skeletal muscle of pre-T2DM patients with obesity increased after 16 wk of RT[11]; however, no change in GLUT4 level in the gastrocnemius muscle of T2DM rats was observed in an experiment involving the simulation of RT using an advanced vertical ladder (80° ladder, 50 times/wk) over 7 wk[12]. McMillin *et al*[13] also demonstrated that rats with skeletal muscle-specific *GLUT4* gene knockout did not experience glucose transport impairment in the plantar muscle after 5 d of muscle load (RT model). The above results suggest that RT seems to increase the content of GLUT4 in the skeletal muscle of patients with obesity and/or T2DM and contributes to the improvement of IR; nevertheless, GLUT4 is not the only factor in the RT-induced increase of glucose transport in skeletal muscle. According to existing research results, other types of GLUT, such as GLUT1 and GLUT10, may be involved, which needs to be confirmed through further studies[14].

RT DELAYS SKELETAL MUSCLE LOSS AND INCREASES MUSCLE MASS

Skeletal muscle is an important tissue for glucose storage and metabolism. With a decrease in skeletal muscle volume and muscle strength, the number of insulin receptors, insulin sensitivity, and muscle utilization of blood-derived glucose decrease, and this readily induces IR and impaired glucose tolerance[15]. Delaying skeletal muscle loss increases insulin sensitivity[16]. According to the 2011 National Health and Nutrition Survey report, muscle mass reduction was positively correlated with IR and HbA1c levels and negatively correlated with glucose metabolism. There was a significantly negative correlation between muscle strength and IR index in patients with T2DM[17]. RT can significantly increase skeletal muscle strength and increase the cross-sectional area of the quadriceps femoris[18], thus increasing the number of insulin receptors and improving insulin sensitivity. After 8 wk of RT, the upper arm muscle mass and relative muscle strength of patients with T2DM were increased, and the levels of fasting blood glucose and fructosamine were significantly decreased[19]. We observed that resistance and aerobic exercises can significantly increase limb muscle content and body fat content and significantly reduce blood glucose and HbA1c in patients with T2DM[20]. RT can increase muscle mass more effectively than aerobic training[21]. The increase in muscle mass induced by RT is related to the increase in protein synthesis in skeletal muscle cells. The main pathway of muscle

protein synthesis is the insulin-like growth factor-1 (IGF-1)/PI3K/Akt/mammalian rapamycin target (mTOR) pathway. RT can upregulate the expression of IGF-1 and eventually activate mTOR[22] to promote mRNA translation and protein synthesis. West *et al*[23] discovered that the activities of IGF-1 and mTOR in lower limb muscles of female rats were significantly increased after intervention with RT.

RT REDUCES THE ACCUMULATION OF LIPIDS IN SKELETAL MUSCLE

Lipid accumulation in skeletal muscle cells can lead to IR. The specific mechanism includes the following two aspects. First, intracellular free fatty acids and their potential metabolites can be used as signal molecules to activate protein kinases, such as protein kinase C, JUN kinase, and nuclear factor inhibitor kinase B. These kinases can phosphorylate serine on the insulin receptor substrate (IRS), thus inhibiting the IRS-mediated insulin signal transduction pathway. Finally, it causes IR in skeletal muscle cells and other peripheral target cells. With the continuous development of mass spectrometry, bioactive lipids, such as diacylglycerol, sphingomyelin, and sphingosine glycerol, are more likely to be related to the mechanism of IR[24]. New evidence suggests that the localization of triglycerides, diglycerides, and sphingomyelin seems to play an important role in promoting the reduction of insulin sensitivity[25]. Transmission electron microscopy revealed that submuscular intramyocellular triacylglycerol (IMTG) was negatively correlated with insulin sensitivity of type II fibers, whereas IMTG between muscle fibers was either positively or not correlated with insulin sensitivity. Early work involving diacylglycerol (DAG) in the Bergman Laboratory demonstrated that DAG located on the skeletal muscle membrane was negatively correlated with insulin sensitivity, whereas cytoplasmic DAG was not related to insulin sensitivity[24]. RT or acute RT can affect the accumulation of lipids in muscle[26,27], and this reduction in intracellular lipid accumulation is beneficial in mitigating peripheral IR. It was found that insulin sensitivity increased even after RT[28]. Insulin sensitivity recovered 2 h after RT, and intramyocellular lipid content either did not change[29] or returned to the basic level[30] The utilization rate of intramyocellular triacylglycerol (IMTG) in resistance movement seems to depend partly on the initial concentration; therefore, the higher the content of IMTG, the higher its degradation rate. However, the mechanism still requires further investigation. The effects of acute RT on bioactive lipids (such as DAG and sphingolipids) and other polar lipids (such as acylcarnitine) are unclear. Some scholars believe that exercise can improve the coordination of the transfer, intake, and oxidation of free fatty acids, thus reducing the accumulation of lipids in skeletal muscle cells[31].

Second, free fatty acids can directly activate Toll-like receptor 4 to trigger the natural immune response, thus inducing IR through the inflammatory response pathway. Toll-like receptors are a family of highly conserved proteins that play an important role in the recognition of microbial pathogens. The activation of this receptor signaling pathway can increase the expression of proinflammatory factors and play an important role in the mediation of systemic inflammation. In recent years, studies have shown that high inflammation of skeletal muscle can damage the transduction of the insulin receptor signal, which may play an important role in the occurrence and development of IR[32]. In patients with T2DM, 16 wk of aerobic exercise combined with RT significantly decreased the levels of C-reactive protein, interleukin (IL)-1 β, IL-8, and tumor necrosis factor-α and increased the levels of anti-inflammatory factors, such as IL-6, IL-10, and IL-15[33], thus promoting the conversion of white fat to brown fat[34] and playing a positive regulatory role in insulin secretion and islet β-cell proliferation. However, it is not clear whether this effect has long-term stability. The effects of 12 wk of aerobic exercise combined with RT on cardiovascular risk factors in male adolescents with obesity were observed. It was revealed that aerobic exercise combined with RT could significantly reduce the levels of serum IL-1Ra, interferon-γ-inducible protein 10a, insulin, and the homeostasis model assessment of IR index in men with obesity[35]. Recent studies have reported[36] that RT potentially increases muscle mass and systemic insulin sensitivity, reduces inflammatory response, increases adiponectin, and improves related metabolism in patients with T2DM.

RT IMPROVES MICROVASCULAR BLOOD FLOW IN SKELETAL MUSCLE

The main function of microvascular units is to provide a site for the exchange of various reaction substrates, oxygen, and hormones between plasma and extracellular

fluid in the tissue. At rest, approximately 30% of the skeletal muscle capillaries are perfused. Studies have revealed that changing the endothelial exchange area of skeletal muscle can affect the transport of insulin to and its effect on skeletal muscle[37]. The increase in skeletal muscle microcirculation perfusion caused by many factors, including insulin, mixed meals[38], angiotensin II receptor inhibitor, muscle contraction, and others, is related to the increase in skeletal muscle insulin uptake. This demonstrates that microcirculation plays a very important role in insulin uptake by skeletal muscle. According to Russell *et al*[39], after systemic RT for 6 wk, IR and fasting blood glucose in patients with T2DM were significantly lower than those before exercise. Further, they were significantly correlated with the increase in microvascular blood flow (MBF) stimulated by RT and independent of the change in muscle capillary density. It is suggested that the increase in MBF induced by RT is beneficial in improving the level of glucose metabolism in patients with skeletal muscle IR and T2DM. Zhao *et al*[40] discovered that the blood-flow velocity of the low-load blood-flow restriction regimen was significantly higher than that of traditional RT after exercise, which may be related to the stimulation of shear force and metabolic stress during exercise. This enhanced vascular compliance and accelerated tissue perfusion as well as metabolic product transport and clearance after exercise.

CONCLUSION

In conclusion, RT predominantly improves insulin sensitivity in skeletal muscle through the following mechanisms: Increasing muscle mass, increasing the expression of GLUT4 in skeletal muscle, reducing the accumulation of lipids and inflammation in skeletal muscle, and increasing MBF. Therefore, it can be used in the prevention and treatment of T2DM. In order to obtain the expected clinical effect, the exercise type, duration, and intensity of RT require further study.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 April 28; 9(2): 108-127

DOI: 10.13105/wjma.v9.i2.108

ISSN 2308-3840 (online)

REVIEW

Is COVID-19-induced liver injury different from other RNA viruses?

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Author contributions: Al-Nimer MS contributed to the concept, data collection, editing and revision of this manuscript.

Conflict-of-interest statement: Authors declare no conflict of interest for this article.

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Manuscript source: Invited manuscript

Specialty type: Infectious diseases

Country/Territory of origin: Iraq

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C

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Abstract

Coronavirus disease 2019 is a pandemic disease caused by a novel RNA coronavirus, SARS coronavirus 2 (SARS-CoV-2), which is implicated in the respiratory system. SARS-CoV-2 also targets extrapulmonary systems, including the gastrointestinal tract, liver, central nervous system and others. SARS-CoV-2, like other RNA viruses, targets the liver and produces liver injury. This literature review showed that SARS-CoV-2-induced liver injury is different from other RNA viruses by a transient elevation of hepatic enzymes and does not progress to liver fibrosis or other unfavorable events. Moreover, SARS-CoV-2-induced liver injury usually occurs in the presence of risk factors, such as nonalcoholic liver fatty disease. This review highlights the important differences between RNA viruses inducing liver injury taking into consideration the clinical, biochemical, histopathological, postmortem findings and the chronicity of liver injury that ultimately leads to liver fibrosis and hepatocellular carcinoma.

Key Words: Liver injury; COVID-19; RNA-viruses; Risk factors; Liver enzymes; Liver fibrosis

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Core Tip: Coronavirus-induced liver injury is rare, and it may be passed without a definite diagnosis. Liver function tests are simple, inexpensive and rapid and can detect acute liver injury, particularly in infected patients who have evidence of comorbidities. This review discusses the differences between RNA virus-induced liver injury focusing on the coronavirus targeting the liver as an extrapulmonary site of infection or as a part of multiple organ dysfunction

Citation: Al-Nimer MS. Is COVID-19-induced liver injury different from other RNA viruses?

Grade D (Fair): D
Grade E (Poor): 0

Received: January 23, 2021
Peer-review started: January 23, 2021
First decision: February 28, 2021
Revised: March 12, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Huang CH, Sergi C, Zhang W
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Ma YJ



World J Meta-Anal 2021; 9(2): 108-127
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/108.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.108

INTRODUCTION

The genetic material of RNA viruses is usually single-stranded RNA and occasionally double-stranded. Examples of single-stranded RNA viruses are rhinoviruses, influenza viruses, coronaviruses, dengue viruses, hepatitis viruses, west Nile viruses, Lassa virus, Ebola virus, Rabies lyssavirus, polioviruses and measles morbillivirus. Examples of double-stranded RNA viruses are rotaviruses and picobirnaviruses. According to the polarity of the viral envelope, they are grouped into positive-sense and negative-sense (Figure 1). Coronaviruses are RNA viruses that belong to the coronaviridae family. These viruses are enveloped with a positive single-stranded RNA genome sized 26.4-31.7 kilobases[1]. The genome of coronaviruses is larger than other RNA viruses (Figure 1).

In humans, coronaviruses cause mild respiratory symptoms (*e.g.*, rhinoviruses) or lethal respiratory stress syndrome [*e.g.*, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and coronavirus disease 2019 (COVID-19)]. In mice, coronaviruses cause hepatitis and encephalomyelitis[2,3]. Extrapulmonary organ dysfunction is also reported by coronavirus infections in humans. Dysfunction of the hepato-biliary system is an uncommon clinical presentation of coronavirus infections.

The liver is one of the extrapulmonary organs attacked by COVID-19[4-6]. Some SARS coronavirus 2 (SARS-CoV-2)-infected people showed abnormal liver function tests, and other patients showed liver injury as a late sequel of multiple organ dysfunction or failure[7]. Abnormal liver function tests, including increasing levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactic dehydrogenase are observed[8]. Serum bilirubin level is also reported to be increased in some COVID-19 patients at the time of admission into the hospital[9].

High abnormal serum levels of aminotransferases were observed in severe illnesses rather than in trivial or mild COVID-19 illnesses[10]. There are several characteristic features of high serum levels of aminotransferase enzymes. The first feature is a transient and slight increase of serum ALT and AST levels. The second feature is a dynamic pattern of liver enzymes: liver dysfunction started with an elevation of AST, slight changes of total bilirubin levels followed by increased ALT in severe patients[11], and the pattern of cholestasis is absent[8,12]. Liver failure and bile duct injuries were not pathological features of COVID-19[13]. Therefore, any elevated transaminase enzyme is considered an indirect expression of systemic inflammation, and there are no specific symptoms that have been linked to liver dysfunction[13]. The third feature is the elevation of aminotransferase enzymes is related to race as Chinese patients less frequently had high serum aminotransferases compared with United States patients infected with COVID-19[14]. These enzymes (ALT, AST, alkaline phosphatase and γ-glutamyl transpeptidase) are increased with increased disease severity. The mortality rate increased by 14.87 fold in patients with AST level (40-120 U/L), and the male gender is positively associated with elevation of AST[11].

The fourth feature is that ALT among many biochemical markers was studied as a predictor of COVID-19 severity using univariate and multivariate ordinal logistic regression models on 598 patients presenting with moderate, severe and critical illness. The results showed that patients with ALT > 50 U/L had worse severity of illness (odds ratio: 3.304, 95% confidence interval: 2.107-5.180)[15]. Serum ALT level is a stronger predictor of disease severity compared with myohemoglobin, but it is weaker than cardiac troponin I or aging and the presence of high blood pressure.

Phipps *et al*[16] categorized the acute liver injury in patients with coronavirus according to the serum levels of ALT into mild [ALT > the upper limit normal (ULN) < 2 times ULN], moderate (ALT 2-5 times the ULN) and severe (ALT > 5 times the ULN). Those authors found that 45% had mild, 21% moderate and 6.4% severe liver in a total number of 2273 patients with positive SARS-CoV-2 tests[16]. The category of severe acute liver injury had significantly high inflammatory markers and a higher rate of mortality that accounted for 42%[16]. In one cohort study that included 176 patients, 109 (61.9%) had evidence of liver dysfunction, and only 34 out of 109 (31.2%) had an acute liver injury (ALT and/or AST ≥ 3 ULN), which was associated with an increase in serum bilirubin, lactic dehydrogenase and C-reactive protein[17].

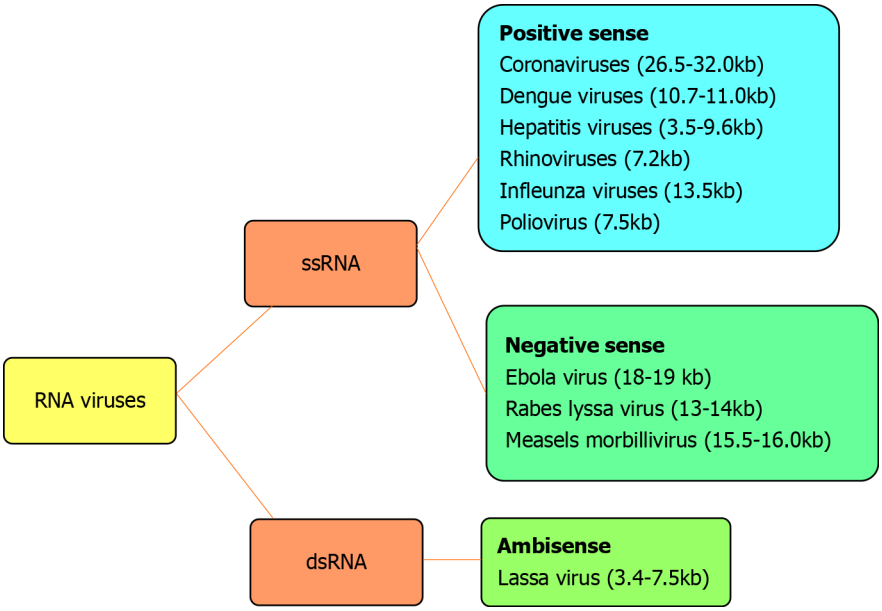


Figure 1 Distribution of the RNA viruses according to their strands and senses. ssRNA: Single-stranded RNA; dsRNA: Double-stranded RNA.

Another meta-analysis that included 3428 patients collected from 20 studies found that severe COVID-19 is associated with increasing levels of serum AST, ALT and bilirubin and a decreasing serum albumin level[18]. This study showed no evidence of bias of all studied variables as the calculated *P* value of the Egger's test was nonsignificant ($P > 0.1$).

The fifth feature is that there is no clear association with adult respiratory stress syndrome. Most studies link acute liver injury with respiratory stress syndrome with hypoxemia and multiple organ failure. The sixth feature is they may be associated with an increased serum bilirubin level. In one study that included 11245 patients, the majority of patients presented with elevated serum bilirubin (9.7%), AST (23%), ALT (21.2%), gamma-glutamyl transferase (GGT) (15.5%) and alkaline phosphatase (ALP) (4%)[19]. Patients presenting with higher levels of serum bilirubin at admission indicate the progression of the disease severity[20,21].

The seventh feature is that high levels of aminotransferases are not a specific effect as other serum levels of enzymes related to the heart, kidney and muscle are also increased The eighth feature is that the high serum aminotransferases are infrequently associated with a slight increase of GGT, ALP and bilirubin[14]. During admission the serum level of ALP, an index of cholangiocyte injury, is within normal limits and several factors are associated with increased serum levels of ALP including male gender, increased neutrophil count, corticosteroid use and antifungal drugs[11]. The final feature is that SARS-CoV-2-infected children showed minimal or no increase in the hepatic enzymes[22].

The explanation of abnormal serum levels of ALT and AST are attributed to several factors. SARS-CoV-2 infected the pulmonary tissue causing hypoxemia. As a result of hypoxia-reoxygenation injury, Kupffer cells are activated leading to the generation of the free radicals that induce acute liver cell injury[23,24]. Hypoxia *de novo* is a form of stress that increases the activity of the sympathetic autonomic nervous system and releases adrenocorticotrophic hormone from the hypothalamus leading to acute liver injury[25]. In addition, flaring of the intestinal microflora in the critical COVID-19 cases causes septic shock, which induces hypoxia of the hepatocytes and thereby hepatocellular necrosis and cholestasis[26].

Abnormal ALT and AST serum levels are also attributed to direct transmission of SARS-CoV-2 from the bowel to the liver[13,27]. Inflammation and high levels of cytokines associated with SARS-CoV-2 infection causes multiple organ dysfunction. Biomarkers related to the inflammation are independent risk factors of developing acute liver injury in COVID-19. Interleukins (IL), including IL-1β, IL-6 and IL-8, and soluble tumor necrosis factor (TNF) receptor-1 are increased and played a role in multiple organ dysfunction[28]. Serum C-reactive protein ≥ 20 mg/L and lymphocyte count < $1.1 \times 10^9/mm^3$ are significantly associated with acute live injury[7].

Chronic liver diseases also cause abnormal levels of AST and ALT. Patients with viral hepatitis are more susceptible to SARS-CoV-2 infection. Lei *et al*[11] reported 84 patients out of 5771 had a previous history of liver disease (4 patients had fatty liver, and 77 patients had viral hepatitis. Sarin *et al*[29] reported that elevation of serum bilirubin and AST/ALT ratio predicted a poor prognosis of COVID-19 (mortality rate of 43%) in patients with liver cirrhosis. On the other side, a recent meta-analysis study that included 17 studies showed that chronic liver disease had no significant effect on the poor prognosis of COVID-19[30].

Drug-induced hepatocyte injury can also cause abnormal AST and ALT levels[31,32] (Table 1). Accordingly, drug-induced acute liver injury referred to an elevation of ALT by ≥ 5 ULN, or an elevation of ALP (in the absence of bone diseases) by ≥ 5 ULN, or the elevation of ALT by ≥ 3 ULN plus elevation of serum bilirubin level by > 2 ULN[33,34]. Drug-induced liver injury is either idiosyncratic or intrinsic in nature, and several risk factors played a role in inducing liver injury (Figure 2). Antiviral agents with potential hepatotoxic effects, *e.g.*, remdesivir, lopinavir and tocilizumab should also be considered[35-37]. Acetaminophen, which might cause liver damage, is usually used in the management of COVID-19 to reduce body temperature[38,39]. Azithromycin therapy is a possible cause of acute liver injury in COVID-19[40].

STRUCTURAL CHANGES IN THE LIVER DUE TO SARS-COV-2 INFECTION

Several studies clarified that SARS-CoV-2 causes nonspecific hepatic changes according to the postmortem histopathological and immunohistochemistry studies. The histopathological changes observed in the liver autopsies are[15,41] microvascular steatosis, lobular focal necrosis, cellular neutrophil, lymphocyte and monocyte infiltration in the portal area, microthrombosis and hepatic sinuses congestion. There is no evidence suggesting liver failure or development of granuloma. Electron microscopy studies showed that the endothelial cell contained coronavirus particles[42]. The RNA sequence data findings and immunohistochemistry studies showed expression of angiotensin-converting enzyme-2 receptors (ACE-2R) in the cholangiocytes but not in Kupffer cells[43], which may not be related to COVID-19. In normal liver, immunohistochemistry showed expression of ACE-2R in the bile duct lining cell but not in the hepatocytes.

COVID-19 AND METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE

Metabolic dysfunction-associated fatty liver disease (MAFLD) is the acronym of nonalcoholic fatty liver disease (NAFLD) as NAFLD is associated with components of the metabolic syndrome, including obesity, diabetes mellitus and dyslipidemia[44]. Therefore, COVID-19 and metabolic dysfunction-associated fatty liver disease/NAFLD can affect each other. For example, when the patients with metabolic dysfunction-associated fatty liver disease/NAFLD are infected with SARS-CoV-2, their livers are susceptible to the harmful effects of the drugs that are clinically used in the management of the COVID-19[45-47]. Also, patients with metabolic derangement are more susceptible to SARS-CoV-2 infection (*e.g.*, diabetes mellitus)[48]. Diabetic patients already have asymptomatic or symptomatic liver dysfunction or injury.

A recent study on 202 COVID-19 patients showed NAFLD is an independent risk factor for the progression of COVID-19 with an ultimately poor prognosis[49]. The most common pattern of liver damage reported in the Ji *et al*[49] study is mild hepatocellular injury, while the ductular or mixed with hepatocellular injury was observed in 2.6%. Moreover, persistent abnormal liver function tests were observed in 33.2%, and NAFLD was independently associated with the progression of liver injury in 19.3% of COVID-19 patients. The percentage of liver injury induced by SARS-CoV-2 is related to the associated risk factor rather than the specificity of SARS-CoV-2 to attack the liver directly because ACE-2R is absent in Kupffer cells and available in 3% of the hepatocytes[43,50].

Acute liver injury was reported in 19 out of 187 (15.4%) patients in one study performed at the Seventh Hospital of Wuhan City, China. Five of the patients had a cardiac injury represented by a higher serum level of troponin[51]. Zhang *et al*[52] summarized the studies that showed an abnormally high level of ALT, AST and GGT, which is a diagnostic marker for cholangiocyte injury, was found to be elevated in 30

Table 1 Antiviral drugs with a promising efficacy against coronavirus disease 2019 with potential hepatotoxic effect

Drug category	Efficacy and hepatic adverse reactions
Protease inhibitors: lopinavir, ritonavir	They are effective against SARS and MERS but not against COVID-19. These drugs failed to improve the clinical manifestations or shorten the time of conversion to negative test of viral nucleic acids in the bronchoalveolar fluid specimens. They caused increase serum bilirubin and aminotransferases levels in COVID-19 patients.
Viral RNA-dependent RNA polymerase: remdesivir, favipiravir	(1) Remdesivir is an adenine nucleotide analogue used in the management of Ebola virus infection. It inhibits the activity of viral RNA-dependent RNA polymerase. It improves the clinical manifestations of COVID-19 patients presenting with hypoxia with low blood oxygen saturation. It causes a significant increase in AST/ALT in COVID-19 patients; and (2) Favipiravir is a guanosine analogue with a broad-spectrum antiviral activity similar to remdesivir. It is effective against the clinical manifestations and shortening the recovery period of moderate COVID-19 illness. Experimental studies showed that favipiravir increases the serum levels of AST, ALP, ALT and total bilirubin and increased vacuolization in hepatocytes.
Membrane fusion inhibitors: umifenovir	Umifenovir is an indole derivative molecule with broad-spectrum antiviral activity. It is effective in the management of COVID-19, particularly when combined with protease inhibitors. The data are very limited about the hepatotoxicity of umifenovir. Chronic users had high serum transaminases.
Interferon lambda	Endogenous INFλs potentiate the long-term immunity by stimulating T helper 1, cytotoxic T cell and antibody response. It prevents the development of a cytokine storm. Recombinant INFλ may be a promising therapy for COVID-19. There is no clinical evidence that INFλ induces liver injury.

COVID-19: Coronavirus disease 2019; SARS: Severe acute respiratory syndrome; MERS: Middle East respiratory syndrome; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; INFλ: Interferon lambda.

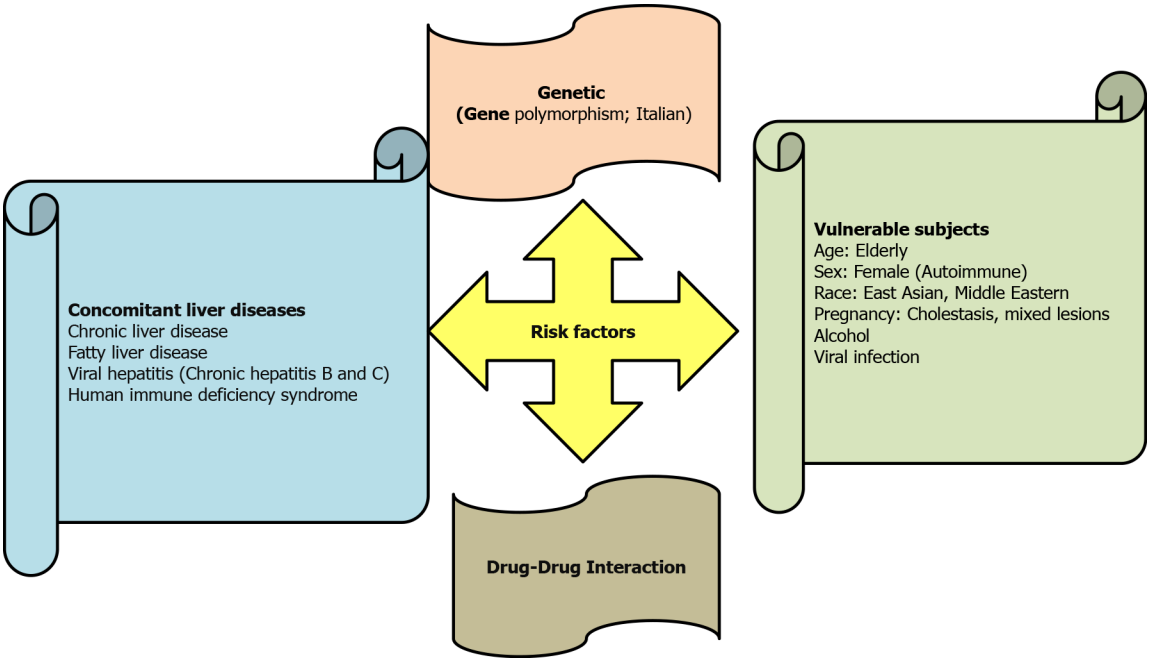


Figure 2 Risk factors of drug induced liver injury.

of 56 patients with COVID-19 during hospitalization. The other important trigger factor of the progression of COVID-19 is drug-induced liver injury. Patients with fatty liver are at a higher risk for drug-induced liver injury compared with noninfected healthy individuals or patients without fatty liver. Some drugs might cause severe liver injury in obese patients with COVID-19, while others might induce the transition of fatty liver to nonalcoholic steatohepatitis (NASH) or worsen the preexisting pathological liver lesion[53].

SARS-COV-2 INFECTION AGGRAVATES THE PROGRESSION OF NAFLD

Hepatocellular hypoxia is the most common explanation of hepatic metabolic derangement induced by SARS-CoV-2 infection, which leads to increases of ACE-2R in liver tissue. Tissue hypoxia is a feature of COVID-19 that is followed by an insidious and paradoxical pattern. In experimental studies, ACE-2R protein was significantly

increased in the liver tissue after bile duct ligation, and the activity of ACE-2R is also increased in the human cirrhotic liver compared with a healthy liver[50]. An *in vitro* experimental study showed that hypoxia increased the expression of ACE-2R in CD34 cells derived from mice subjected to hind-limb ischemia without alteration in the activity of ACE[54]. Hepatocellular hypoxia also increases the expression of inducible hypoxic factors. These factors play a role in the adaptation of the endothelial cell to the hypoxia by inducing the expression of genes that promote energy metabolism of the cells[55]. These factors have dual effects as HIF-1α induces inflammation by upregulating NF-kB and CD4+, CD8+ and producing proinflammatory cytokines (IL-2 and TNF-α)[56,57].

Hepatocellular hypoxia activates reactive oxygen and nitrogen species. Extensive release of cytokines (cytokines storm) play roles in recruiting the immune cells to the site of inflammation, producing vasodilation and increasing the vascular permeability and production of nitrative and oxidative free radicals, which collectively cause tissue damage[7,58-60]. A nitrative stress syndrome in COVID-19 is evoked by IL-2 and IL-6[7,61], while oxidative stress syndrome was expressed in COVID-19 by IL-6 and TNF-α, which increase the levels of superoxide anion in neutrophils and hydrogen peroxide[62-64]. Antioxidants are useful in the management of COVID-19, *e.g.*, vitamin C, Nrf2 activators, zinc, glutathione and N-acetylcysteine[65-67]. It is important to mention that macrolides are prescribed in the management of COVID-19 because they (*e.g.*, azithromycin and erythromycin) inhibit the generation of superoxide anion and nitric oxide[68-70].

High inflammatory response of Kupffer cells is linked to hepatocellular hypoxia. Kupffer cells are part of innate immunity that acts as scavengers and phagocytes. During COVID-19 illness, the ACE-2Rs are expressed in the circulating inflammatory macrophages and tissue macrophages, including Kupffer cells[71]. Expression of these receptors may cause Kupffer cell dysfunction and lead to acute liver injury. Yuan *et al*[72] reported that Kupffer cells have dual actions against viral hepatitis as they cleared the hepatitis B virus (HBV)/HCV due to their phagocytic activity and at the same time produce inflammatory mediators that eventually cause immune tolerance towards HBV/HCV.

COVID-19 AND CHRONIC LIVER DISEASE

In one study including 28 patients presenting with variable chronic liver disease of different etiological factors, it was found that patients with acute on chronic liver failure showed worse outcomes, and a poor outcome was observed in patients managed with mechanical ventilation[73]. The mortality rate was 33% in patients with decompensated liver cirrhosis, while it accounted for 8% in patients with chronic liver disease without cirrhosis[73]. The therapeutic regimen of hydroxychloroquine, remdesivir, other antivirals and plasma therapy did not improve the outcome of COVID-19 in patients with chronic liver disease. Moreover, acute liver injury was reported in 14 out of 105 (13.3%) patients with coinfections of COVID-19 and viral hepatitis due to HBV. Because 4 out of 14 (28.57%) patients progressed within a short time to acute on chronic liver failure, and the study concluded that liver injury in patients with SARS-CoV-2 and chronic HBV coinfection was associated with severity and poor prognosis[74]. Transient elevations of ALT, AST, GGT, ALP and bilirubin were observed in a small number of coinfected patients with COVID-19 and HBV[74].

COVID-19 AND LIVER TRANSPLANTATION

Patients with liver transplantation are at risk of infection with COVID-19. Maggi *et al*[75] reported 16 patients managed with liver transplantation showed positive COVID-19 laboratory tests. Moreover, the mortality rate in long-term liver transplantation survivors infected with COVID-19 was 2.7% (3 out of 111 survivors), which is comparable to those without liver transplantation[76]. Drug-drug interaction should be considered in the liver transplant recipient, particularly between immunosuppressive antiviral therapeutic regimens. A combination of ritonavir and protease inhibitors should be avoided, while chloroquine and remdesivir are safe[77].

ANTIVIRAL AGENTS AND LIVER INJURY

During the COVID-19 pandemic, a number of specific antivirals drugs are indicated in the management of severe COVID-19 or patients at risk of developing severe disease. These drugs are listed in Table 1.

Ritonavir (a protease inhibitor) can induce hepatotoxicity by laboratory evidence of elevation of aminotransferases[78,79] and clinically presented with jaundice and hepatomegaly. In addition, ritonavir can induce dyslipidemia characterized by increasing serum levels of triglyceride and low-density lipoprotein cholesterol, while the serum level of high-density lipoprotein cholesterol is decreased[78,80,81]. Long-term therapy can cause dyslipidemia, which is a risk factor for developing NAFLD. Therefore protease inhibitors are unlikely to cause dyslipidemia because they are used for a short period in the management of COVID-19.

Lopinavir is another protease inhibitor used for COVID-19. It caused severe liver injury in HIV-infected patients[82]. The combined therapy of ritonavir and lopinavir leads to increased toxicity of ritonavir, which presented with a higher elevation of transaminases[83]. It is important to mention that protease inhibitor-induced hepatotoxicity is not related to lopinavir plasma levels but to ritonavir, which accumulated in the liver[82]. A combination of lopinavir/ritonavir does not show significant improvement in the clinical and laboratory testing of COVID-19; indeed it may produce adverse reactions[84].

Remdesivir is an adenosine nucleotide analog with broad-spectrum antiviral activity used for RNA virus infection treatment[85]. The most common hepatotoxicity of remdesivir in COVID-19 patients is the elevation of hepatic transaminases and multiple organ failure[86]. The elevation of ALT and AST is usually reversible.

Favipiravir (6-fluoro-3-hydroxy-2-pyrazine carboxamide) is a guanosine analog with a broad-spectrum antiviral activity similar to remdesivir. It selectively inhibits the RNA-dependent RNA polymerase of the virus and has a broad-spectrum activity against influenza virus and all RNA viruses that cause hemorrhagic fever[87]. According to the evidence-based studies, favipiravir is effective against clinical manifestations and shortening the recovery period of moderate COVID-19 illness[88]. In SARS-CoV-2-infected patients, favipiravir significantly increased the ALT and AST enzymes. Experimental studies showed that favipiravir increases the serum levels of AST, ALP, ALT and total bilirubin and increased vacuolization in hepatocytes.

Chloroquine and hydroxychloroquine have antiviral activity and have been recommended as the second therapy choice in the treatment of COVID-19[89]. Limited data were available concerning the hepatotoxicity of chloroquine.

Umifenovir (Arbidol) inhibits the adsorption of the virus to the host cell and thereby prevents viral penetration of the cell. It is effective against influenza, hepatitis C and SARS-CoV-2 infections[90-92], and it has a broad therapeutic index. Chronic use of umifenovir is associated with increased transaminase levels[93]. A triple combination of lopinavir, ritonavir and umifenovir is considered in the management of COVID-19 patients, but this combination induced liver damage in 50% of patients and elevated hepatic transaminases and bilirubin[94].

Recombinant or pegylated interferon lambda (INFλ) is available endogenously in four moieties and have a potent antiviral activity as they maintain the antiviral response in the pulmonary tissue[95]. Synthetic (recombinant) INFλ has been effective against viral replication and obviates the development of a cytokine storm[96,97]. In an experimental animal model of SARS-CoV-2 infection, the production of INFλ is decreased. Therefore, administration of synthetic recombinant INFλ can restore the endogenous INFλ and improve the immune system.

OTHER RNA VIRUSES

MERS

MERS-CoV is a viral RNA transmitted from infected camels to humans[98,99]. Severe MERS-CoV infections were observed in immunocompromised patients, diabetes mellitus, renal failure and the elderly age group similar to COVID-19[100,101]. Acute renal failure is the most common extrapulmonary complication in MERS-CoV patients, which accounts for 75%[102,103]. Histopathological findings of the liver autopsies of patients with MERS[104,105] are similar to those observed with COVID-19, which included: (1) mild chronic lymphocytic portal inflammation; (2) mild hydropic degeneration of hepatocytes; (3) mild sinusoidal lymphocytosis; (4) no evidence of lobular or portal granuloma or fibrosis; (5) mild steatosis; and (6) occasional

intralobular hemorrhage and necrosis.

Ultrastructural findings detected by electron microscopy showed MERS-CoV inside the macrophage, while SARS-CoV-2 was detected in the cytoplasm of the hepatocyte.

SARS

In SARS-CoV, the elderly age group who had the liver disease of whatever etiology showed the highest mortality rate[106]. Postmortem liver autopsy studies revealed that SARS-CoV particles were present in less than 50% of liver tissue with approximately 1.6×10^6 copies/g hepatic tissue[107]. The characteristic features of liver injury in SARS are cellular apoptosis, increased mitotic activity, balloon degeneration of the hepatocyte, lymphocytic cell infiltration and central lobular necrosis[105,108]. Infrequently, fatty degeneration was observed[109]. Real-time PCR of the liver tissue was positive (similar to SARS-CoV-2), but electron microscopy did not show virus particles[107,110].

HIV and liver injury

Liver disease is the third most common cause of death in HIV patients, which is mainly due to HIV/HCV coinfections[111]. Several factors are contributed to causing death in HIV patients, including metabolic syndrome, consumption of alcohol and coinfections with hepatitis C and D viruses[111]. HIV patients are more likely to have fatty liver (alcoholic or nonalcoholic), which can present with any clinical manifestations. Therefore, these patients have clinical and laboratory evidence of metabolic syndrome with and without a high body mass index[112]. NAFLD with HIV infection is usually present as NASH and progresses to liver fibrosis and cirrhosis. The laboratory technique of elastography discloses the presence of liver fibrosis in 30%-50% of HIV patients[113,114].

The characteristic features of liver diseases in patients with HIV infections are: (1) patients with HIV infection commonly have NAFLD; (2) patients with HIV and NAFLD commonly have NASH compared with those who do not have HIV infection[115,116]; (3) an HIV-infected person with NAFLD had a lower body mass index compared with patients without HIV infection despite the similarities between the two groups in the other components of metabolic syndrome[117]. Moreover, HIV-infected persons with NAFLD had a higher percentage of visceral fat compared with uninfected HIV persons[118-120]; (4) previous studies showed that antiretroviral medicines are not a cause of NAFLD, and they did not play any role in the pathogenesis of NAFLD in HIV-infected patients. HIV-infected patients managed with highly active antiretroviral therapy for 12 mo showed significant hepatotoxicity when the patients had a higher baseline serum AST level and a lower serum albumin level. Hepatotoxicity presented with elevated serum transaminases and a significantly high AST:ALT ratio[121]. One retrospective study of HIV-infected patients found that concomitant viral hepatitis, duration of antiretroviral drugs and baseline data of liver enzymes can increase the risk of liver damage in HIV-infected patients[122]; (5) cytokines, including IL-6 and TNF-α, are not responsible for inflammation that progressed to liver fibrosis and cirrhosis[123]; (6) liver fibrosis commonly occurred in patients with concomitant diseases, *e.g.*, viral hepatitis C and HIV despite confounding factors, including using antiretroviral drugs and heavy alcohol consumption[124]; (7) in HIV-mono-infected patients, significantly high AST and ALT were found in 5.6% and 16.8%, respectively, while hepatic steatosis and fibrosis were found in 55.0% and 17.6%, respectively[125]. Middle aged males with evidence of metabolic syndrome were more vulnerable to liver fibrosis in HIV mono-infected persons; (8) noncirrhotic portal hypertension is an uncommon complication of HIV-infection[111]; (9) hepatic granuloma and hemosiderosis and chronic active hepatitis were reported in 16%, 26% and 3%, respectively. The higher percentage of hepatic granuloma is related to intravenous drug abuse in HIV patients, while the low incidence of chronic active hepatitis is attributed to T lymphocyte depletion[126]; (10) histopathological changes of the liver showed a nonspecific or pathognomonic feature that indicated HIV infection. One study including 42 liver autopsies found that histopathological changes are usually secondary to infection, inflammation, cirrhosis and cancer[127]; and (11) HIV patients with secondary specific hepatic infections have higher serum ALP and AST compared with noninfected HIV patients[126].

Influenza A virus and liver injury

Influenza A virus is an enveloped, single-stranded RNA virus with eight RNA fragments. Infected patients with influenza A/Kawasaki/86 (H1N1) showed significant elevation of the serum aminotransferases indicating viral hepatitis. The

significant high serum liver enzymes usually occurred after the fever had subsided, which indicates that the viral replication is not the cause but the consequent activation of the immune response[128]. Rarely, using chloroquine in the prevention of influenza can cause hepatitis (elevated serum amino transaminases)[129].

H1N1 differed from the seasonal influenza infection by inducing hepatocellular injury with increased serum AST and ALT levels[130,131], while infection with H7N9 can induce hepatic hypoxia and fatty infiltration[132,133]. In experimental studies using mice, influenza A virus types H1N1, H5N1 and H7N2 significantly increased serum hepatic enzymes on day 5 postinfection, and the immunohistochemistry showed positively infected hepatocytes with viruses[134]. In one Korean population study, the relationship between the H1N1 2009 pandemic and acute viral hepatitis A showed a significant inverse (r = -0.597) relationship, *i.e.* as the prevalence of hepatitis A virus increased the prevalence of H1N1 2009 decreased. This indicates that the hepatitis A virus (positive-stranded RNA virus) damped the virulence of H1N1 2009 to attack the liver and produce exacerbation of the acute liver injury by a mechanism still unknown[135].

Viral hepatitis and liver injury

Hepatitis A virus (HAV) produces a broad spectrum of liver injury varied from mild illness (a transient elevation of serum transaminases and bilirubin) to fulminant hepatic failure, and the HAV-RNA particle was detected in the serum of the infected patient[136]. Blood donor participants with previous HAV infection showed a high serum level of ALT and anti-HAV antibody, while the HVA-RNA was not detected in the serum[137]. Infected patients with hepatitis E virus have low serum levels of ALT compared with HAV infection, while the serum level of GTT is high, and the patients clinically are asymptomatic[138]. Infected patients with HCV may have a previous history of HBV infections, and their illness linked with liver autoimmune diseases as autoantibodies are detected in their sera[139]. Most HCV are mono-infections, and a minority have HCV and HBV coinfections at a single time point with a complex pattern of virological profiles. In patients with positive hepatitis B surface antigen, the estimated prevalence of HBV/HCV is approximately 5%-20%, while in HCV positive antibody patients is 2%-10%, considering the geographical distribution factor[140]. Patients with chronic hepatitis B surface antigen are liable to be infected with HDV[141]. Chronic hepatitis D patients showed high viral load and transaminase levels and progressed rapidly to liver cirrhosis[142].

The relationship between serum lipids and viral hepatitis are complex. There is evidence that in the acute phase of viral hepatitis, the serum triglycerides are elevated and abnormal lipoproteins are detected in the serum[143]. A low serum level of cholesterol and apolipoprotein A indicated a severe liver injury[143]. NAFLD is usually associated with HBV and HCV infection, and the reason for hepatic steatosis is related to the existence of the components of metabolic syndrome (*e.g.*, obesity, diabetes mellitus, dyslipidemia, *etc.*). Hepatic steatosis is related to the HCV-genotype 3 infection, which exerts direct metabolic effects on the liver[144]. Chronic HBV and HCV infections lead to liver cirrhosis and hepatocellular carcinoma, which carries a poor liver function compared with NASH-induced hepatocellular carcinoma[145]. A case report study mentioned a 24-year-old patient presented with mild COVID-19 disease, and he experienced a cytokine storm due to coinfection with HBV leading to multiple organ dysfunction syndrome and death[146]. This case report highlights the worsening prognosis of superinfection with viral hepatitis in patients with RNA virus infection with coronaviruses and HIV.

Dengue virus

The clinical manifestations of the dengue virus are dengue fever, dengue with plasma leakage, dengue hemorrhagic fever and dengue shock syndrome. The liver is the target of the dengue virus infection, and the severity of the infection is usually assessed by laboratory investigations, including serum aminotransferases, albumin and blood count in addition to abdominal ultrasound[147-152]. There is evidence that the dengue virus regulates hepatic lipids in order to replicate inside hepatocytes[153], leading to an increase of aminotransferases and thrombocytopenia[154-156]. Previous experimental studies showed that intracellular lipid droplets are essential for dengue virus replication, and postmortem studies confirmed that lipid vesicles in the liver autopsies are used for dengue virus replication[151,157].

NAFLD is a feature of dengue virus infection, whether related to the clinical category of dengue with or without plasma leakage[158]. A high serum ALT level is a characteristic feature of dengue virus infection during the febrile period of any clinical category[158]. Patients with dengue hemorrhagic fever complicated with multiple

organ failure showed elevation of both ALT and AST, while those patients without multiple organ failure did not show elevation of the serum AST level[159]. Fatty infiltration in dengue-infected patients with plasma leakage indicating dengue severity as hemoconcentration and thrombocytopenia are commonly reported in those patients[158]. Postmortem histopathology studies of liver autopsies of dengue-infected patients showed sinusoid congestion, hepatocytes necrosis, fatty infiltration or steatosis[160].

Ebola virus and liver injury

Ebola virus disease (formerly called Ebola hemorrhagic disease) is a disease caused by the RNA virus transmitted from animals (*e.g.*, bats) to humans and spreading through human-to-human transmission. It is a fatal disease complicated with multiple organ dysfunction like COVID-19[161]. The fatality rate of Ebola virus disease is attributed to the exaggeration of the immune response leading to extensive production of cytokines (cytokine storm) like COVID-19[162]. Hepatomegaly and hepatocellular necrosis are the characteristic features of liver lesions induced by the Ebola virus. Bradfute *et al*[163] demonstrated that Ebola virus induced-hepatocyte apoptosis is linked to the progression of the disease, while lymphocyte apoptosis is not involved in the progression of the disease. Patients who recovered from Ebola virus disease may complain from recurrent hepatitis in the future[164,165].

Lassa virus and liver injury

Lassa virus is an old-world arenavirus that is transmitted from small animals, *e.g.*, rat, to humans. In 1969, the virus was first described in Nigeria, and it caused fatal hemorrhagic fever. Lassa fever can cause hepatitis, which was not the cause of death or progressed to liver failure[166]. Lassa virus-induced liver injury is acute active hepatitis without lymphocytic infiltration, which progresses to more hepatocyte necrosis or recovery. Gross and histopathological findings of liver autopsies are fatty metamorphoses but no evidence of fatty infiltration like COVID-19 and hepatocellular necrosis with eosinophilic, not lymphocytic, infiltration[167]. In an experimental hamster model, Pirital virus produced similar changes observed in human Lassa fever, including elevation of serum levels of hepatic transaminases combined with hepatocellular necrosis[168].

ROLE OF RNA VIRUSES IN LIVER FIBROSIS

Liver fibrosis is a term for a progressive accumulation of the extracellular matrix leading to disruption of the normal liver architecture[169,170]. This condition develops in response to viral infection, metabolic disorders, chemicals, heavy alcohol consumption and autoimmune diseases[171,172]. At the molecular level, HBV through HBV X protein, which is expressed during infection, will activate the hepatic stellate cells[173] and induce fibrosis, while hepatitis delta virus is involved in the progression of existing fibrosis[174]. Hepatitis delta virus antigen (notably the large isoform) activates the HBV X protein to mediate and enhance the signals that induce liver fibrosis. On the other hand, proteins derived from HCV are *de novo* profibrogenic [175]. This indicates that HCV-induced liver fibrosis mechanisms differed from that mentioned with HBV or HCV. Moreover, some authors believe in the role of oxidative stress, mitochondrial dysfunction and iron accumulation in the pathogenesis of liver fibrosis[176-178]. HIV is not fibrogenic *per se*, but it can accelerate existing liver fibrosis induced by HBV or HCV[179]. Mono-HIV infected persons do not significantly show liver fibrosis compared with those presenting with coinfections with HBV or HCV[180]. Figure 3 summarizes the liver injury induced by RNA viruses and shows that the outcome event of liver fibrosis or cirrhosis is not a feature of COVID-19.

CONCLUSION

Acute liver injury is an asymptomatic manifestation of COVID-19 represented by a transient and reversible increase of liver enzymes. Microvascular steatosis, lobular focal necrosis, immune cell infiltration in the portal area and microthrombosis are postmortem findings of patients with multiple organ dysfunction. The liver injury produced by SARS-CoV-2 infection has completely differed from the corresponding liver damage induced by other RNA viruses. Risk factors of liver injury in COVID-19

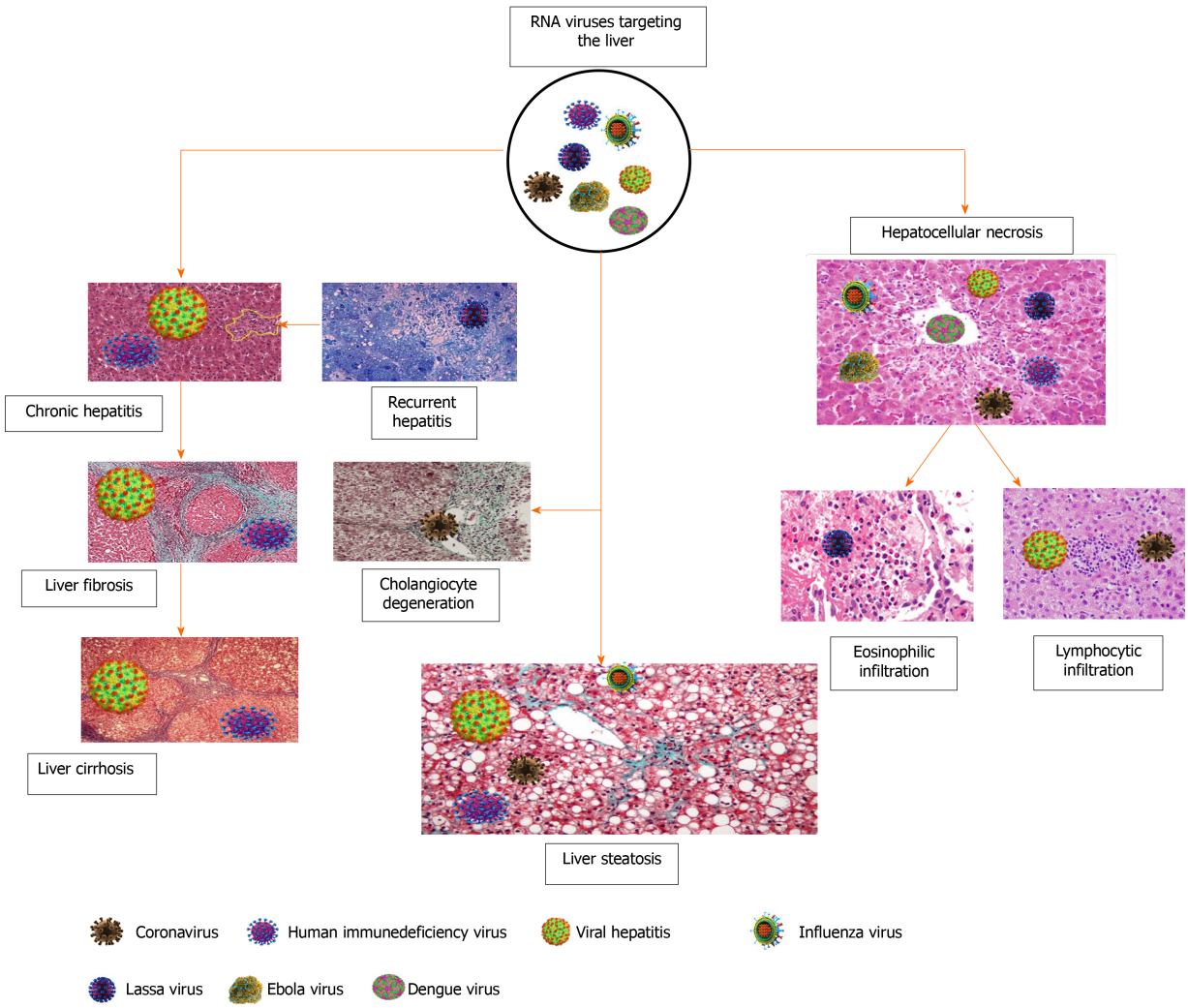


Figure 3 Liver injury categories induced by RNA viruses.

patients are age, race, concomitant liver diseases and evidence of metabolic derangement. The chronicity of liver injury is still unknown, and further prospective studies are recommended to clarify the role of SARS-CoV-2 in inducing fibrosis and fatty liver.

ACKNOWLEDGEMENTS

The author expresses his gratitude to Professor Dr. Ismail I. Latif, the Dean of the College of Medicine, University of Diyala for his kind support, and to Assistant Lecturer Sura K. Khubala at University of Imam Ja'afar Sadiq for the technical support.

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World J Meta-Anal 2021 April 28; 9(2): 128-138

DOI: 10.13105/wjma.v9.i2.128

ISSN 2308-3840 (online)

MINIREVIEWS

Ankle injuries in athletes: A review of the literature

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Author contributions: Jona James J contributed to the literature reviewing, writing and editing of the manuscript; and Al-Dadah O conceived the original idea and design of the study and extensively revising the manuscript critically for important intellectual content. All of the authors reviewed and approved the final manuscript as submitted.

Conflict-of-interest statement: We confirm that all authors have no conflicts of interest.

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Manuscript source: Invited manuscript

Specialty type: Orthopedics

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Abstract

Ankle injuries are commonplace in the athletic population, with lateral ligament sprains accounting for the majority of them. The medial ligament complex, the distal tibiofibular syndesmosis as well as any of the bones that constitute the ankle joint can also be injured. Typical mechanisms of injury include inversion-plantar-flexion and external rotation on a supinated, dorsiflexed or pronated foot. Lesions of the ankle present with similar symptoms of pain, swelling and tenderness. Therefore, a thorough history and physical examination must be obtained to make the correct diagnosis. This is especially critical for athletes as certain injuries can lead to termination of their career if not treated accurately on time. Imaging may be useful in some cases to confirm or rule out differential diagnoses. Most injuries can be managed conservatively using the Protection, Rest, Ice, Compression and Elevation protocol followed by a comprehensive rehabilitation programme. Surgery is reserved for grade III ligament tears that are refractory to initial non-operative treatment and displaced fractures that are unlikely to unite without surgical intervention. The objective of this review is to discuss the common ankle injuries encountered in the athletic population and the approaches to their diagnosis and management.

Key Words: Ankle sprain; Athlete; Deltoid ligament; Lateral ligament; Syndesmosis; Fracture

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Core Tip: This is an up-to-date literature review on the topic of ankle injuries that encompasses both ligamentous injuries as well as fractures.

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): E

Received: January 3, 2021
Peer-review started: January 3, 2021
First decision: March 8, 2021
Revised: March 26, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Huo Q, Liu P, Yamakado K
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li X

Citation: Jona James J, Al-Dadah O. Ankle injuries in athletes: A review of the literature. *World J Meta-Anal* 2021; 9(2): 128-138
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/128.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.128

INTRODUCTION

The health and social benefits of undertaking exercise are extensive. However, sports are the second highest cause of injuries after home and leisure accidents, and a major cause of pain, loss of mobility, disability and even death[1]. The incidence of ankle injuries in the general population is 1 in 10000 per day, but this number increases to 5.23 in athletes, with up to 9.35 ankle injuries per 10000 athletes during competition[2,3]. Ankles are the most common site of sports injuries; lateral ligament sprains account for 76.7% of injuries followed by fractures at 16.3%[4,5]. High incidences of sprains were seen in team sports such as soccer and rugby, which necessitate quick directional changes whilst running. In contrast, fractures were frequent in high-impact sports like trampolining, parachuting and mountaineering[3,4]. Less frequently, syndesmotic injuries, or "high" ankle sprains, which occur above the ankle joint, are reported. These usually occur in sports that are high-speed and high-impact and take place on uneven surfaces, for example, downhill skiing, football and basketball[4,6].

The purpose of this review is to provide an overview of the common ankle injuries occurring in the athletic population and the approaches to assessing and managing them.

ANATOMY

The talocrural, or "true" ankle joint, is articulated by the tibia and fibula of the lower leg and the talus of the foot. It is a synovial hinge joint allowing dorsiflexion and plantarflexion of the foot relative to the lower leg. Stabilising the joint further are the medial and posterior malleoli of the tibia and the lateral malleolus of the fibula. The talocalcaneal, or subtalar joint, is directly below the ankle joint and is composed of the talus and the calcaneus. This joint enables inversion and eversion of the foot[7].

The ligaments of the ankle are divided into the lateral collateral ligaments, the medial (deltoid) collateral ligaments and the syndesmotic ligaments (Figure 1)[7]. The lateral ligament consists of three separate ligaments: the anterior talofibular ligament (ATFL), the posterior talofibular ligament (PTFL) and the calcaneofibular ligament (CFL)[2,7]. The ATFL and CFL originate from the lateral malleolus and insert into the talar neck and calcaneus, respectively. Whilst the ATFL works to limit pathological inversion and plantar flexion, the CFL restricts over-inversion of the ankle joint[8]. The PTFL arises from the malleolar fossa of the lateral malleolus and inserts into the posterior talus. It is under tension when the foot is in dorsiflexion and relaxed when the foot is in plantarflexion or in a neutral position. It is the strongest of the lateral ligaments, and therefore isolated PTFL injuries are rare unless there is complete ankle dislocation. The PTFL does not have an independent role in maintaining ankle stability when the other lateral ligaments are intact. However, if the other ligaments are ruptured, the short and long fibres of the PTFL restrict external rotation of the talus, talar tilt and dorsiflexion, with the short fibres also limiting internal rotation[9].

The deltoid ligament has four parts superficially: tibionavicular, tibiocalcaneal, tibiospring and the posterior tibiotalar ligaments. These cross the ankle and the subtalar joint attaching to the medial malleolus proximally and to the talus, calcaneus and navicular distally. The deep components comprise the anterior tibiotalar ligament and the deep posterior tibiotalar ligament, which only cross the ankle joint. The superficial layers restrict talar abduction and the deep layers prevent external rotation. Both layers work together to limit pronation of the talus[10]. The distal tibiofibular syndesmosis binds the tibia and fibula and is held together by four major ligaments: the anterior inferior tibiofibular ligament, the posterior inferior tibiofibular ligament, the transverse tibiofibular ligament and the interosseous ligament. This syndesmosis complex is crucial to maintaining ankle stability and resisting forces that would otherwise separate the tibia and fibula and widen the ankle mortise[6-8,11]. This arrangement of ligaments provides static as well as dynamic congruity[12]. The

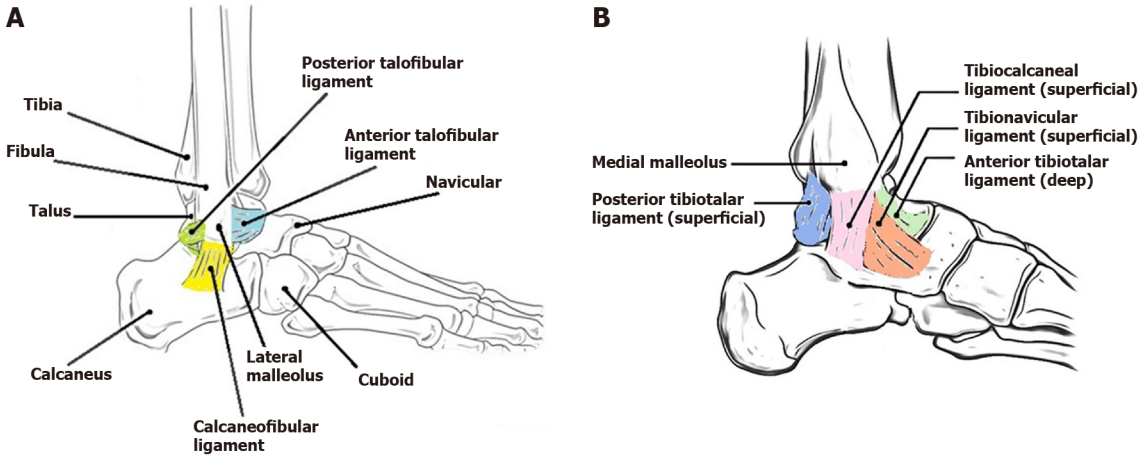


Figure 1 Lateral and medial view of the ankle. A: Lateral view of the ankle showing anatomy of the ankle joint and the lateral collateral ligaments (Adapted from Wikipedia[43]); B: Medial view of the ankle showing the medial collateral ligaments (Adapted from Earth's Lab[44]).

movements of the ankle joint have been summarised in Figure 2.

LATERAL LIGAMENT ANKLE SPRAINS

Mechanism of injury

The term ankle sprain is used to describe a range of pathology from an overstretched ligament to complete tearing of the ligaments of the ankle joint[13]. In the majority of cases, the mechanism of injury involves inversion of the plantarflexed weight-bearing foot[13,14]. Lateral ligaments are most commonly damaged; in 65% of cases, the ATFL alone is injured as it resists inversion and is the weakest of the three lateral ligaments[2,15]. Less commonly, both the ATFL and the CFL are affected, whilst injury of the PTFL is rarer and only due to severe ankle joint dislocation[15].

History

A previous lateral ligament sprain is a risk factor for recurring ones and is often reported when a history is undertaken[16]. Pain is the major symptom, located laterally over the ATFL area[17]. The gold standard for diagnosing lateral ligament injury is delayed physical examination of the ankle joint[13,18]. In the hours and days immediately following the injury, pain and tenderness are felt diffusely and haematoma has not developed, so diagnosis is not accurate. However, after 4 to 5 d, the location of pain and tenderness on palpation, plus the characteristic haematoma and swelling can be localised. The specificity and sensitivity at this stage is 84% and 96%, respectively, in deducing the presence or absence of an ATFL injury[18].

Physical examination

Two provocative tests can be performed to assess the stability of the ankle joint, both of which must be compared with the uninjured leg: the anterior drawer test and the talar tilt test[15]. These are best done a few days after the injury when the patient can be relaxed and in less pain[17]. An anterior drawer test evaluates the integrity of the ATFL. The patient is seated with the knee joint flexed to 90° to relax the calf muscles, and the ankle joint in a 10°-15° plantarflexed position. With the tibia held firmly, the calcaneus is pulled forward. An absent or poor endpoint due to increased anterior talar displacement combined with laxity of the joint indicates a positive test finding. In 50% of cases, a dimple sign can be seen over the area of the ATFL during the manoeuvre. The talar tilt test assesses the integrity of the CFL. The patient is supine, and the ankle is in a neutral position. With the heel braced and both the talus and calcaneus held, the heel is inverted relative to the tibia. A positive test finding is indicated when a firm endpoint cannot be established, and there is increased joint laxity[15,17].

Investigations

Radiographic analysis is not typically indicated for ankle sprains as a physical examination is deemed sufficient. Magnetic resonance imaging (MRI) if used has 75%-

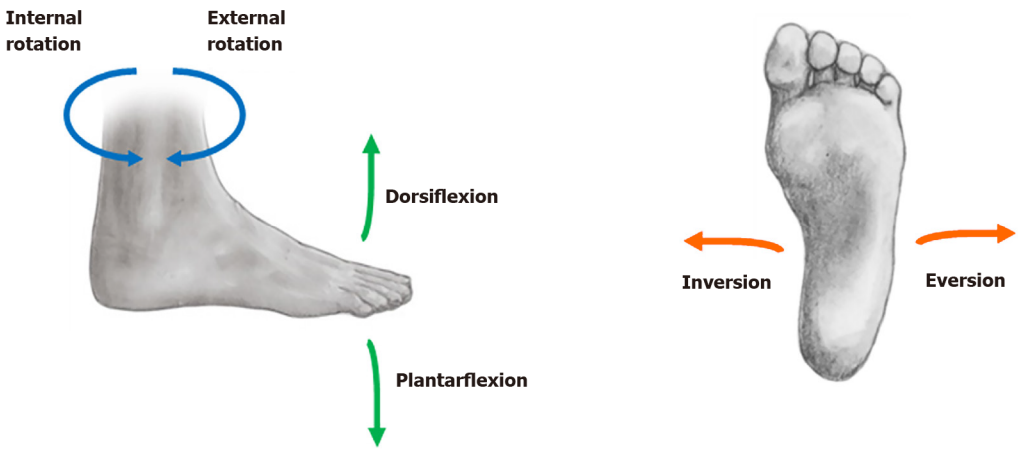


Figure 2 Ankle joint planes of motion (Adapted from CrossFit[45]).

100% sensitivity in diagnosing lateral ligament sprains. However, its high costs combined with the high incidence of ankle sprains render its use uneconomical[15]. Occasionally an X-ray is required as determined by the Ottawa Ankle Rules criteria to rule out a fracture[17].

Classification

Ankle sprains can be categorised anatomically and functionally according to severity. Grade I injuries are where the ATFL has been stretched and may have microscopic tearing. Mild swelling occurs, and walking is possible with minimal pain. Grade II injuries are caused by a stretched ATFL, and sometimes CFL, where there is partial tearing of the fibres. There is moderate swelling and ecchymosis, and walking is painful. Grade III injuries are the most severe as the ATFL, CFL and PTFL are injured, and the ankle joint is unstable. Severe immediate swelling and ecchymosis is seen, and the intense pain makes it difficult to bear weight[2,17,19]. A superior approach to grading ankle sprains divides them into stable and unstable injuries depending on the outcomes of the anterior drawer test and talar tilt test. If both tests are negative, this is a stable grade I injury. A positive anterior drawer test makes it an unstable grade II injury, whereas if both tests are positive, it is classed as an unstable grade III injury. Although this system only accounts for injury to the ATFL and CFL, its reproducibility and ease of use make it ideal for use in the Emergency Department[15]. It should be noted that grading is highly subjective with much debate about the classification systems, and differences in inter-rater agreement have been observed[17]. Return-to-play (RTP) for grade I injuries is between 1 to 2 wk whilst grade III injuries take 4 to 6 wk longer.

Nonoperative management

The majority of ankle sprains can be managed with conservative treatment. For mild-moderate injuries, protection of the ankle joint with functional support coupled with progressive exercise is thought to be superior to immobilisation[7,18,20]. Cast immobilisation may cause muscle stiffness and wasting whilst functional support such as ankle tape and/or tubular elastic bandage allows a quicker RTP and less residual pain[15,21,22]. Moreover, semi-rigid support such as an Aircast Air-Stirrup ankle brace (DJO Global Inc., Vista, California, United States) has been shown to provide significant improvement in ankle joint function at 10 d and 30 d compared to elastic support bandage[21]. For stable grade I and II injuries, the Protection, Rest, Ice, Compression and Elevation (PRICE) treatment method is recommended to reduce swelling and inflammation alongside functional support and early exercise, which concentrates on increasing range of motion[2,15]. Grade II injuries may require additional orthotic support like the Aircast for a longer period of up to 6 wk. Thereafter, a rehabilitation programme including peroneal strengthening and proprioceptive exercises should be followed to enable quicker recovery and RTP[2]. Early mobilisation allows healing by stimulating the collagen bundle orientation[7]. As grade III injuries are unstable, immobilisation for up to 1 mo is advised alongside strengthening exercises. Generally, in the acute phase, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) leads to a significant reduction in pain[23]. However, one study reported that the swift return to activities that NSAIDs provide might

impair the healing process, as trial participants who were given piroxicam had greater swelling and instability 14 d post-injury[20]. Evaluation of the effects of therapeutic ultrasound in the treatment of acute ankle sprains shows limited evidence[24].

Surgical intervention

Whilst most patients heal successfully, those with persistent symptoms past 3 mo despite nonoperative management should be evaluated for chronic lateral ankle instability. This is primarily managed with physical therapy and bracing. However, surgery is indicated in those who show no improvement after 3-6 mo[2,7,15,25]. Surgery is typically performed as an outpatient procedure under general anaesthesia with a popliteal nerve block. The surgical procedures are classed as anatomical or nonanatomical reconstruction. Anatomical reconstruction includes the Broström and modified Broström procedures whereby the ATFL and CFL are shortened and reinserted. These are excellent in restoring ankle stability and provide longevity. Nonanatomical reconstruction involves tendon transfers, such as the Watson-Jones, Evans and Chrisman Snook procedures. These surgical techniques have the potential postoperative risks and complications of functional instability and early arthritis and can also limit range of motion[2,25].

MEDIAL LIGAMENT ANKLE SPRAINS

Mechanism of injury

Sprains of the medial ligament of the ankle joint are uncommon and are typically found to be in conjunction with fibular fractures and sprains of the tibiofibular syndesmosis[15,17]. Only around 4% of all ankle ligament injuries are due to injury to the deltoid ligament[26]. The usual mechanism of injury tends to be a pronation-external rotation position of the foot, though pronation-abduction and supination-external rotation positions are also observed[10,17]. These injuries tend to occur in sports such as basketball, dancing and long jump[26].

History

Pain, tenderness and swelling are present on the medial side of the ankle, and ecchymosis may present a few days later. Weightbearing will be difficult on the affected foot and may be associated with a feeling of instability. Some patients also report a popping sensation or a feeling of the foot ‘giving way’ especially when walking downhill[5,10,17].

Physical examination

When examining the affected ankle, it is important to check for signs of fractures and syndesmotic sprains as these commonly present alongside deltoid ligament injuries. If the ligament is ruptured completely, this may be palpated beneath the medial malleolus. During the external rotation stress test, deltoid ligament damage is indicated if the talus moves away from the medial malleolus[10,17,27,28].

Investigations

Standard weight-bearing radiographs in the anteroposterior (AP), lateral and mortise views may provide further clues to aid diagnosis. A width of 4 mm or higher of the medial clear space between the talus and medial malleolus indicates disruption of the deltoid ligament and syndesmosis. Isolated deltoid injuries do not cause widening of the medial clear space as the lateral malleolus will hold the talus in place[5,10,17].

Classification

Deltoid ligament sprains are categorised by the extent of involvement of the ligament. Type I lesions are a proximal tear, type II lesions involve the intermediate component, and type III lesions are distal injuries of the deltoid ligament[10,26,29].

Nonoperative management

Similar to lateral ligament sprains, the PRICE protocol is the recommended method of management for medial ligament ankle sprains. Immobilisation in a cast with additional support from bracing or taping further facilitates recovery. Following this, a rehabilitation programme focussing on building strength and improving coordination and proprioception is advised. Return to training at light intensity can be expected in 6-8 wk[10,27].

Surgical intervention

Surgery is not routinely indicated for isolated deltoid ligament injuries[27].

SYNDESMOTIC ANKLE SPRAINS

Mechanism of injury

Unlike lateral ankle sprains, syndesmotic ankle sprains are thought to be caused due to external rotational forces acting on a dorsiflexed foot. The exact mechanism cannot be determined as the movement occurs too quickly to be sufficiently recognised. In this position, the ligaments typically injured are the anterior inferior tibiofibular ligament and the interosseous ligament depending on the everted or neutral position of the foot, respectively[6,7,11,20]. Isolated syndesmotic ankle sprains are relatively unusual, as they are usually associated with fibular fractures and/or deltoid ligament sprains. Syndesmotic injuries can lead to functional limitations and chronic pain, so prompt diagnosis and treatment are crucial[11,30].

History

Pain is the principal complaint, localised to the anterior inferior tibiofibular ligament and often exacerbated by active and passive external rotation and/or dorsiflexion of the foot. Upon palpation, the ankle is tender in this area. Pain may also be felt proximally due to damage to the interosseous membrane or more serious injury. Swelling is significantly less than that for lateral ligament sprains. However, poor/absent weight-bearing and/or plantar flexion is seen[11,15,17].

Physical examination

Three tests can be undertaken to determine injury to the syndesmosis: the squeeze, crossed-leg and external rotation stress test (Kleiger's test). In the squeeze test, the foot is dorsiflexed, and the midshaft of the tibia and fibula are squeezed together. For the crossed-leg test, the patient rests the distal third of the fibula of the affected leg on the knee of the opposite leg. A gentle downward compressive force is then applied to the knee of the injured leg. To perform the external rotation test, the patient is seated. The tibia is then stabilised as the foot is dorsiflexed and rotated externally relative to the tibia. During all three manoeuvres, pain over the syndesmotic area indicates a positive sign[15,17,30].

Investigations

As syndesmotic injuries are so rare, the above tests have only been validated in a small group of patients. Their sensitivity and specificity cannot be reliably determined. Therefore, imaging is recommended in this scenario[15]. Standard ankle weight-bearing radiographs in the AP, mortise and lateral views should be performed to rule out fractures and determine syndesmosis widening[31]. If syndesmosis widening is seen or there is proximal fibular tenderness, then full length radiographs of the tibia and fibula in the AP and lateral views are required to exclude a Maisonneuve fracture. There is no consensus on the guidelines indicating syndesmosis diastasis. However, a joint space widening of greater than 5 mm or tibiofibular overlap of less than 10 mm on AP view is frequently used. Stress radiographs in the lateral view with the foot externally rotated in both dorsiflexion and plantarflexion may also show the diastasis and any posterolateral displacement of the fibula[11,17]. MRI is highly sensitive (100%), specific (93%) and accurate (96%) in determining the extent of syndesmotic injury[6,15,30]. A high-intensity signal resembling the Greek letter lambda (λ) is seen on a coronal MRI, known as the 'lambda sign'. Although not a diagnostic indicator in itself, in combination with positive physical examination findings, the lambda sign can help clinicians determine which patients require surgical intervention[32]. MRI can also demonstrate secondary findings such as a bone bruise, osteochondral lesions and anterior talofibular ligament injury, all of which are associated with poorer response to rehabilitation and longer recovery periods. Arthroscopy shows promise for its use as a diagnostic tool; one study has found it to be 100% accurate in identifying syndesmotic injury[11].

Classification

The classification system for syndesmotic ankle sprains grades injuries from I to III by severity, similar to lateral ligament sprains. Athletes should be informed that recovery times for syndesmotic ankle sprains are up to two times longer than that for lateral

ligament sprains, especially if managed conservatively[6,8]. One study found that the time to RTP and/or functional disability was directly correlated to the degree of tenderness of the interosseous membrane[33].

Nonoperative management

Conservative treatment is the standard management for athletes who have a sprain without diastasis. Initially, PRICE is recommended to reduce pain and associated symptoms. Whilst lateral ligament sprains benefit from early mobilisation, syndesmotic injuries are more severe and therefore require nonweight-bearing immobilisation and stability such as bracing, walking boots and casting during the acute phase. This allows healing, protects the syndesmosis from further injury and prevents further rotation of the talus and fibula. As the pain subsides, protected weight-bearing training can begin using crutches. Light balance training on a balance pad and low-level strength training using Theraband (The Hygienic Corp., Akron, Ohio, United States) can commence. Exercises such as seated heel raises can also help with strengthening. As the patient recovers, functional exercises such as double to single heel raises and hops are added to the rehabilitation program. The final stage involves progression from walking fast to jogging, running and sport-specific training. Ankle braces and/or taping must be used for stability when returning to play[6,11,20,30,31].

Surgical intervention

Surgery is indicated for patients who have diastasis of the syndesmosis with or without concurrent fibular fracture and for those with lower grade injuries that are refractory to conservative treatment. The syndesmosis has to be reduced initially, and this can be done open, closed or arthroscopically and checked with fluoroscopy. Two surgical methods are commonly used. In syndesmotic screw fixation, screws are placed in position to hold the syndesmosis in place until healing can occur. The postoperative plan includes being nonweight-bearing for 6-8 wk, and the functional outcomes are excellent if the syndesmosis is reduced adequately. There is no significant difference between routinely retaining or removing syndesmotic screws as both groups of patients have similar clinical outcomes, and such a removal is not necessarily routinely recommended. If screw breakage occurs in situ over time, they may be removed in symptomatic patients after 8 to 12 wk from the original surgical procedure. However, for the vast majority of patients, breakage of the screws will have little clinical consequence[34].

Generally, one or two cortical screws are inserted above the ankle joint going through three or four bone cortices in an anteromedial direction (fibula to tibia) to avoid malreduction. At present, there is no consensus in terms of number of screws used or cortices purchased, screw diameter size or the exact location of the fixation. It also depends on patient variables such as a history of osteoporosis and smoking and degree of syndesmotic instability. Two screws are usually preferred as they offer greater resistance. The screw type can be cortical, which is fully threaded, or cancellous, which is fully or partially threaded. Cortical screws are used for positioning such as with plate fixation whilst cancellous screws are useful as lag screws, exerting a compressive force. There is no strong evidence to support placement through three *vs* four cortices[6,31,34,35]. The Syndesmosis TightRope XP implant fixation system (Arthrex Inc., Naples, Florida, United States) uses a strong suture loop that is tensioned between two buttons attached to the tibia and fibula. There is no risk of hardware failure or need for device removal with this method. Furthermore, studies report that the functional outcomes are equal or superior to that achieved with screw fixation and that TightRope fixation allows earlier RTP[6,30].

ANKLE FRACTURES

Mechanism of injury

In sports, ankle fractures generally occur alongside ankle sprains and therefore result from a wide variety of trauma. The commonest mechanism of injury is an external rotation force acting on a supinated foot transferred through the talus to the malleoli[36]. Any of the malleoli can be injured: lateral, medial or posterior. Their fracture pattern can be unimalleolar, bimalleolar (typically lateral and medial) or trimalleolar. Trimalleolar fractures indicate a high energy transfer injury resulting in soft tissue damage and a higher risk of complications. In bimalleolar equivalent fractures, the medial ligament is injured alongside the fibula or the lateral malleolus,

making the ankle unstable. Alternatively, disruption of the distal tibiofibular syndesmosis or a medial malleolar fracture combined with a fracture of the proximal head of the fibula is known as a Maisonneuve fracture. Pilon fractures involve a break in the tibia near the ankle and often the fibula too. The fracture pattern is dependent on the direction of the external force and the positioning of the foot at the time of injury[12,36].

History

Symptoms include immediate severe pain, swelling, bruising, tenderness and an inability to bear weight on the affected foot. If the ankle joint is dislocated, a gross deformity can be seen. A thorough history should be undertaken including mechanism of injury, the position of the foot and the force of impact, all which can help determine the injury patterns and therefore whether ankle stability is compromised. It is important to identify medications. For instance, long-term use of corticosteroids may increase the risk of osteoporosis and fractures. A history of smoking and chronic medical conditions such as peripheral vascular disease and metabolic bone disease should be noted as these affect treatment plans. Other important aspects of the history include occupation if not a professional athlete and living conditions (for example, whether there are stairs in the house)[12,14,36,37].

Physical examination

Along with palpation of the ankle joint and proximal fibula and tibia, an assessment of the neurovascular status and a thorough examination of the soft tissue should be carried out. The joints above and below the ankle joint (knee and foot, respectively) as well as active and passive range of movement of the ankle joint should be checked. A thorough motor and sensory examination is key to determining whether any neurological deficits seen postoperatively are caused due to the fracture or due to iatrogenesis such as a regional nerve block. Tenderness over the proximal fibula suggests a Maisonneuve fracture. Any gross deformities should be reduced as soon as possible under conscious sedation or with an intra-articular block to reduce neurovascular and soft tissue complication risks[12,36].

Investigations

The Ottawa Ankle Rules indicate when an ankle X-ray is required. The rules state that pain in the malleolar zone and one of bony tenderness in the distal 6 cm of the posterior edge of medial malleolus, bony tenderness in the distal 6 cm of the posterior edge of lateral malleolus or inability to bear weight immediately or in the Emergency Department necessitate imaging[38]. X-rays should be standard three view images in AP, lateral and mortise views. The mortise film should be obtained with the foot rotated approximately 15° internally so that the shadow of the tibia on the fibula is eliminated. MRI is not routinely indicated for ankle fractures but may be useful later on to assess concurrent ligamentous injuries. Computed tomography scans are particularly useful in Pilon fractures to further delineate the exact fracture pattern and help facilitate preoperative planning and guide surgical fixation strategies[39].

Classification

There are two classification systems for ankle fractures. The Danis-Weber system is anatomic and categorises fractures based on their location on the fibula in relation to the syndesmosis. Type A fractures are infrasyndesmotic and do not usually cause ankle instability, type B is trans-syndesmotic, and type C is suprasyndesmotic. The Lauge-Hansen system classifies fractures based on the position of the foot and the direction of the force applied to it at the time of injury. Although comprehensive, it does not always reliably predict patterns of injury[12,36].

Nonoperative management

For stable fractures with minor / no displacement, provision of analgesia and cast immobilisation for 6 wk is sufficient to aid healing in most cases. This can slowly be progressed to crutch-assisted weight-bearing and physical therapy once the cast is removed. Throughout the period in cast immobilisation, radiographs are obtained at set intervals (1 wk, 2 wk and 6 wk) to monitor progress[40,41]. Thromboprophylaxis is prescribed in adults during the time the lower leg is immobilised in cast and has been shown to be both clinically effective and cost-effective as it reduces the risk of venous thromboembolism, including both deep-vein thrombosis and pulmonary embolism[42].

Surgical intervention

Unstable and displaced medial and / or lateral malleolar fractures or posterior malleolar fractures greater than a third of the articular surface of the distal tibia with talar tilt and / or talar shift require surgical intervention. This is commonly done by open reduction and internal fixation using a variety of documented surgical techniques with various hardware to achieve anatomic realignment of the fracture. A period of postoperative cast immobilisation is common practice. Thereafter a structured physiotherapy rehabilitation programme to progress the patient back to preinjury level of activity is recommended[37,40,41].

CONCLUSION

The high incidence of ankle sprains and fractures in the athletic population necessitates rapid diagnosis to prevent long-term functional limitations. As these injuries have overlapping symptoms, it is vital to differentiate between them using the history, physical examination and imaging studies. An accurate diagnosis leads to prompt treatment and enables earlier recovery and RTP for the athlete. As the majority of injuries can be treated with the PRICE approach, immobilisation and rehabilitation, surgery is reserved for soft-tissue injuries that are refractory to conservative treatment or displaced and unstable ankle fractures.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 April 28; 9(2): 139-152

DOI: 10.13105/wjma.v9.i2.139

ISSN 2308-3840 (online)

MINIREVIEWS

Viral hepatitis: A brief introduction, review of management, advances and challenges

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Author contributions: The authors were involved in writing different aspects of the draft which were put together and amended by the last author.

Conflict-of-interest statement: None of the authors has any conflict of interest.

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Manuscript source: Invited manuscript

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Abstract

Viral hepatitis represents a major public health hazard and is associated with significant global mortality. Over the last decade, there have been significant developments in the prevention and treatment of viral hepatitis. These changes have led to a situation whereby global elimination has become a realistic goal, fully endorsed by the World Health Organization (WHO). By 2030, the WHO aims to reduce viral hepatitis mortality by 65% and reduce new infections by 90% by 2030. These are ambitious targets and will only be met through a sustained programme. This will require expertise from hepatologists and virologists and the fields of public health and primary care. In this article, we review the causes of viral hepatitis, its management through prevention and treatments, and the most pressing challenges and recent advances.

Key Words: Viral Hepatitis; Management; Direct-acting antivirals; Advances; Challenges

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Core Tip: Viral hepatitis represents a major public health hazard and is associated with significant global mortality. The aim of this article is to provide a concise description of the management, advances and challenges in the field of hepatitis caused by hepatotropic viruses A-E.

Citation: Fagan O, Armstrong P, Merwe KVD, Crosnoi D, Steele C, Sopena-Falco J, Parihar V.

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Ireland

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D, D
Grade E (Poor): 0

Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: February 25, 2021
Revised: February 26, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Masuzaki R
S-Editor: Wang JL
L-Editor: A
P-Editor: Li JH



Viral hepatitis: A brief introduction, review of management, advances and challenges. *World J Meta-Anal* 2021; 9(2): 139-152
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/139.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.139

INTRODUCTION

Viral hepatitis has a high prevalence and is a leading cause of death worldwide, accounting for 1.34 million deaths worldwide in 2015[1]. Lots of different viruses are known to cause liver inflammation, including hepatotropic viruses named A to E. Most of these result in acute self-limiting disease; however, hepatitis B (HBV), HCV, HDV and HEV can become chronic (Table 1). Other viruses (such as Cytomegalovirus and Epstein-Barr virus) can also cause hepatitis as part of a systemic infection. This review considers only hepatitis caused by hepatotropic viruses.

Hepatitis A and E are transmitted through the faecal-oral route by contact with contaminated food or water[2]. In contrast, hepatitis B, C and D are transmitted through bodily fluids[3].

Chronic hepatitis is defined as chronic inflammatory reaction in the liver, as reflected in liver function tests and histology for at least 6 mo[4]. Chronic hepatitis B/C/D/E is defined as the presence of virus (in blood, or in stool for HEV) for more than 6 mo after the new onset of infection. Acute liver failure (ALF) is a syndrome of severe hepatic dysfunction associated with encephalopathy and/or coagulopathy. The American Association for the Study of Liver Diseases (AASLD) define it as "evidence of coagulation abnormality, usually an international normalized ratio above 1.5, and any degree of mental alteration (encephalopathy) in a patient without pre-existing liver disease and with an illness of less than 26 wk' duration"[5]. Globally viral hepatitis remains a large causal contributor to ALF[6] and accounts for the majority of ALF in Asia and Africa[7]. Viral induced ALF is associated with mortality of 18%-91% (with a combined mortality of 50%) in low income countries compared with 3%-45% (with combined mortality of 26%) in upper middle income countries[7].

EPIDEMIOLOGY

HAV

The World Health Organization (WHO) estimated in 2005, 126 million people were affected[8]. Unsurprisingly, the highest rates of HAV infection are found in areas with poor sanitary conditions[9].

HBV

Roughly 30% of the world's population show evidence of current or past infection with HBV[10]. Globally, most HBV infections occur through vertical mother-to-child and early-life horizontal transmission[11].

There are 5 phases of chronic HBV infection[12]. (1) Phase 1: High replicative state with hepatitis B e-antigen (HBeAg)-positive (previously known as 'immune tolerant'). High HBV-DNA levels > 10^6 IU/mL, often normal or slightly increased alanine transaminase (ALT) levels; minimal or no inflammatory changes on liver biopsy; (2) Phase 2: HBeAg-positive chronic HBV. Variable HBV-DNA levels, higher ALT than the previous phase; moderate/severe inflammatory changes on liver biopsy. Can last for months to years; (3) Phase 3: HBeAg-negative chronic HBV (previously known as 'inactive carrier'). Generally, low HBV-DNA levels, normal ALT; the severity of fibrosis depends on the previous stage, but active inflammation is low; (4) Phase 4: HBeAg-negative chronic hepatitis. Variability in HBV-DNA level, ALT and inflammatory changes on liver biopsy. The annual spontaneous clearance of chronic HBV is about 1%[13]; and (5) Phase 5: Occult infection [Hepatitis B surface antigen (HBsAg)-negative]. Undetectable/very low serum HBV DNA with hepatitis B core antibody (Anti-HBc), with or without hepatitis B surface antibody and normal ALT. HBsAg loss before the development of cirrhosis is associated with minimal risk of cirrhosis and improved survival. Immunosuppression may lead to HBV reactivation in these patients.

Table 1 Comparison of hepatotropic viruses hepatitis A-E

Hepatitis	A	B	C	D	E
Viral structure	Naked, ssRNA (Picornavirus)	Envelope, dsDNA (Hepadnavirus)	Envelope, ssRNA (Flavivirus)	Envelope, ssRNA (-ve) (Deltavirus)	Naked, ssRNA (Hepevirus)
Transmission	Faecal-oral	Parenteral, sexual	Parenteral, sexual	Parenteral, sexual	Faecal-oral
Incidence	1.4 million	> 250 million[88]	> 80 million	About 12 million (prevalence)[89]	20 million
Chronic infection	No	About 90% of infants infected; about 2%-6% adult infected	70% (55%-85%)	< 5% of those infected with HBV, > 80% of superinfection)[90]	No (very rarely in immunosuppressed adults)[91]
Other disease associations	None	HCC, cirrhosis	HCC, cirrhosis	Cirrhosis, fulminant hepatitis	None (cirrhosis in chronic HEV infection)
Treatment	Supportive	Viral suppressive therapy (see criteria for commencement of treatment below)	Eradication therapy DAA (gold standard)	PEG-IFN (sparse data)	Supportive

HBV: Hepatitis B virus; HEV: Hepatitis E virus; HCC: Hepatocellular carcinoma; DDA: Direct-acting antiviral.

The HBV genome incorporates into the host genome and is postulated to have an oncogenesis effect contributing to hepatocellular carcinoma (HCC) development[14]. Assay of the novel (or "emerging") biomarker hepatitis B core-related antigen (HBcrAg) is helpful in monitoring patients with chronic HBV infection. Serum HBV DNA correlates with HBcrAg. In patients with undetectable HBV DNA or loss of HBsAg, HBcrAg can still be detected[15]. Decreasing HBcrAg titres are associated with positive outcomes for chronic HBV infected patients[16].

HCV

It is estimated 1.75 million people were newly infected with HCV in 2015[17]. It is the most common bloodborne infection in the USA and the western world[18]. Ninety per cent of HCV infections will progress to chronic liver disease[19]. The most common risk factor for HCV contraction is intravenous drug use, accounting for 80% of cases, with receipt of blood products representing 10.8%[20]. Other less common routes of transmission include organ transplantation, haemodialysis and tattooing.

The WHO estimated that 71 million people were living with chronic HCV infection in 2015[21]. This, however, is not reflected in diagnoses: Only 20% of those who were estimated to be infected were diagnosed in 2016[21], and only 13% of these were treated with direct-acting antivirals (DAAs).

Eighty per cent of HCV infections are found in 31 of 194 countries, with the highest incidences found in Eastern Mediterranean and Eastern European countries[22].

Due to this propensity of the virus to develop into fibrosis, cirrhosis, and HCC, the death rate from HCV is rising despite a reduced incidence. Chronic HCV is often complicated by extra-hepatic diseases such as cryoglobulinemia associated vasculitis, renal disease, and type 2 diabetes[23]. These factors reinforce the importance of treating HCV.

HDV

The incidence and prevalence of HDV is largely uncertain[21]. Some estimate 12 million cases globally; however, there is speculation this could be even higher with cases as high as 60 million[24].

HEV

Twenty million infections of HEV are estimated annually with about 3 million of these causing symptomatic disease[25]. This is predominantly found in south and east Asia, with genotypes 1-4 of 8 affecting humans[26]. This is predominantly transmitted through the faecal-oral route; however, other more recently discovered modes of transmission include blood transfusion, zoonotic infections (through consumption of infected meats) and organ transplants[27].

DIAGNOSIS

The diagnoses of viral hepatitis are primarily made through both serological and molecular assays[28,29]. Serological tests can be either rapid diagnostic tests (field use) or laboratory-based enzyme immunoassays (Table 2).

There is a difficulty in diagnosing viral hepatitis as a cause of ALF. A study in Germany found nearly 50% of cases of HEV causing ALF were misdiagnosed as drug induced liver injuries[30]. Serological testing of HEV alone is often insufficient and molecular testing with polymerase chain reaction needs to be considered more[31].

PREVENTION

Interventions to mitigate contraction of viral hepatitis include sanitation, vaccination, harm reduction policies and safe blood supply practices.

Sanitation

Sanitation has improved worldwide, resulting in reduced annual numbers of diarrheal diseases and of HAV and HEV infections[32].

Vaccination

An effective HAV vaccine has existed since 1992. Adopting wide-scale HAV vaccination depends on socioeconomic conditions. Thirty-four countries have successfully introduced or are planning to introduce universal childhood HAV vaccination as of 2019[33].

The widespread introduction of a universal childhood HBV vaccine (3 doses) has reduced acute and chronic HBV infection worldwide. In 2013, 183 of 194 countries had introduced the HBV vaccine, and worldwide coverage with three HBV vaccine doses was estimated at 81%[34]. The use of antivirals in pregnant mothers with high HBV levels and immunoglobulins with HBV vaccination in infants born within 12hrs can prevent vertical transmission[35,36].

Needle and syringe programmes

Needle and syringe programmes are known to reduce the incidence of HIV without increasing the frequency of persons injecting drugs[37]. The data for reducing HCV incidence are equivocal; as HCV is more efficiently transmitted the aim would be for individuals to inject for the first time in the facility with clean needles and not contract HCV[38].

The advent of standardised HEV testing in meat

HEV infection has increased 10-fold over the past 10 years in Europe. Food-borne infections are linked predominantly with pig meat but also wild boar and deer meat. Currently the only prevention or control option is heat treatment and adequate cooking. Presently the awareness of HEV infection risk associated with undercooked pork is poor and could be optimised[39].

REVIEW OF MANAGEMENT

Management of acute viral hepatitis is mainly supportive except in the instance of ALF, which requires urgent referral to a liver transplant centre to evaluate liver transplantation.

Treatment

HAV: Supportive management is the mainstay of treatment for HAV, which commonly resolves spontaneously.

HBV: When considering management of HBV, one must remember that the virus itself is not cytopathic. Therefore, the clinical outcome following infection depends on the complex interplay between host immune response and viral replication. Acute HBV treatment is mainly supportive. The goal of therapy in chronic HBV is viral suppression to improve/stop liver inflammation and reduce the risk of cirrhosis, HCC and other complications[12,40].

Table 2 Diagnosing hepatitis A-E

Hepatitis	Serological testing	Molecular testing (*via* PCR), quantitative
A	Anti-HAV IgM (acute); Anti-HAV IgG (previous infection, vaccination); Note incubation period of 28 d[92]	HAV RNA
B	Anti-HBc (contact with HBV infection); HBsAg (current infection) appears 1-3 wk post exposure, duration > 24 wk denotes chronicity; Anti-HBS (vaccination, cleared HBV infection); HBeAg (high replication phase (> 10000 IU/mL); Anti-HBe (low replication phase (< 10000 IU/mL); Note incubation 90 d (ranges 45-160 d); Window period 1: first about 8 d of infection; Window period 2: clearance of HBsAg during this period Anti-HBc IgM is detectable[93]	HBV DNA
C	Anti-HCV: Note incubation 6-7 wk (ranges 2 wk to 6 mo)[93]	HCV RNA
D	Anti- HDV IgG	HDV RNA
E	Anti-HEV IgM and IgG and IgA	HEV RNA

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; PCR: Polymerase chain reaction; HBsAg: Hepatitis B surface antigen.

Antiviral choices include tenofovir, entecavir, lamivudine, adefovir and telbivudine[41]. The treatment of chronic HBV depends on multiple factors, including the severity of inflammation/fibrosis, cirrhosis, ALT, E-Ag status, age and family history of HCC. The aim of chronic HBV therapy is the chronic sustained inhibition of viral replication, as curative rates are very low.

Table 3 sets out the recommended European Association for the Study of the Liver (EASL) guidelines for the treatment of those with chronic HBV.

Pegylated interferon-a (PEG-IFN-a) is a first line treatment option in chronic HBV infection. The advantage of PEG-IFN-a over nucelos(t)ide analogues is the finite treatment course, together with superior rates of HBsAg and HBeAg seroconversion[42]. However use of PEG-IFN-a is associated with greater adverse effects including psychiatric, neurologic and endocrinological, effects[43].

HCV: Acute HCV infection often results in mild acute illness lasting 2-12 wk, with less than 25% of infections identified clinically[44]. Eighty to ninety per cent go on to develop chronic HCV infection with 10%-20% of patients developing complications, including cirrhosis and HCC over decades[45]. DAAs are the mainstay of HCV treatment currently (Table 4). DAAs are highly effective at clearing the virus in > 90% of people. Sustained virologic response (SVR) is defined as aviremia 24 wk post completion of antiviral therapy for HCV infection[46]. However, there is some evidence to support the determination of SVR at 12 wk[47]. SVR at 24 wk remains the gold standard. EASL and the AASLD recommend that all HCV patients (both naïve and treatment-experienced) be offered therapy. Different genotypes respond better to different DAAs (Table 5). Pan-genotypic HCV drug regimens, including (sofosbuvir/velaptasvir) and (glecaprevir/pibrentasavir), can be used to treat patients without identifying their HCV genotype, simplifying therapy[48].

HBV/HDV: HDV is a major cause of severe acute and chronic hepatitis[49]. HDV requires the presence of HBV infection for its own viral cycle. HBV/HDV co-infection results in chronicity in 2% of cases; however, superinfection of HDV is associated with 90% chronicity. HDV is a highly pathogenic virus resulting in the most severe chronic hepatitis and the development of cirrhosis within 10 years of infection in 80% of cases[50]. Pegylated interferon can be used as a treatment, although significant data are lacking[51]. Currently HDV treatments are in phase II and III trials.

HEV: HEV infection is usually self-terminating in immunocompetent individuals, typically with detectable serum/stool HEV RNA present for approximately three weeks. In acute self-limiting disease, symptomatic management and management of cholestasis is the primary treatment.

Chronic hepatitis E infection is associated with genotypes 3, 4, 7[27,52]. In endemic areas, the faecal-oral route is the main mode of transmission, while in the developed world food-borne transmission is most common. Most chronic HEV infections are reported in developed countries and thus zoonotic transmission is assumed (through consumption of contaminated meat). Immunocompromised individuals are at an increased risk of contracting chronic HEV infection[53]. The high virulence of genotype 1 may increase the risk of chronic infection. Ribavirin is the agent of choice in

Table 3 European Association for the Study of the Liver 2017 clinical practice guidelines on the management of hepatitis B virus infection[12]

Clinical practice guidelines
All patients with HBeAg-positive or -negative chronic hepatitis B, defined by HBV DNA (2000 IU/mL, ALT) upper limit of normal (ULN) and/or at least moderate liver necroinflammation or fibrosis, should be treated (Evidence level I, grade of recommendation 1)
Patients with compensated or decompensated cirrhosis need treatment, with any detectable HBV DNA level and regardless of ALT levels (Evidence level I, grade of recommendation 1)
Patients with HBV DNA (20000 IU/mL, and ALT) 2 × ULN should start treatment regardless of fibrosis degree (Evidence level II-2, grade of recommendation 1)
Patients with HBeAg-positive chronic HBV infection, defined by persistently normal ALT and high HBV DNA levels, may be treated if they are older than 30 years regardless of the severity of liver histological lesions (Evidence level III, grade of recommendation 2)
Patients with HBeAg-positive or HBeAg-negative chronic HBV infection and family history of HCC or cirrhosis and extra-hepatic manifestations can be treated even if typical treatment indications are not fulfilled (Evidence level III, grade of recommendation 2)

HBV: Hepatitis B virus; HBeAg: Hepatitis B e-antigen; ALT: Alanine transaminase.

Table 4 Comparing pegylated-interferon therapy to nucelos(t)ide analogues therapy

	PEG-IFN	NA
Route of administration	Subcutaneous	Oral
Length of treatment	48 wk	Long-term in chronic HBV (stopping may be considered in some cases[13]); 8-16 wk in HCV
Contraindications	Many (*i.e.*, decompensated disease, comorbidities)	None (dose adjustment according to eGFR)
Tolerance	Inferior tolerability	Excellent tolerance
Side effects	Significant adverse events (psychiatric, neurologic, endocrinological)	Renal impairment (some DAA)[12]
SVR in HCV	40%-75%[94,95]	> 90%[46]
Efficacy in chronic HBV	> 30% HBeAg loss; 3%-5% HBsAg loss[96]	> 11%-18% eAg loss (lower rate of HBeAg seroconversion); Very low HBsAg loss[97]
Cost	Expensive	Expensive when given long-term (*i.e.*, chronic HBV infection)[98]
Resistance	No	Yes

PEG-IFN: Pegylated interferon; NA: Nucelos(t)ide analogues; HCV: Hepatitis C virus; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; DDA: Direct-acting antiviral.

Table 5 First-line recommended direct-acting antiviral therapies for hepatitis C patients

Genotype	1	2	3	4	5/6
DAA schedule (non-cirrhotic)	Sofosbuvir/Ledipasvir; Or Glecaprevir/Pibrentasvir	Sofosbuvir/Velpatasvir	Sofosbuvir/Velpatasvir	Sofosbuvir/Ledipasvir	Sofosbuvir/Ledipasvir
Duration	8-12 wk	12 wk	12 wk	12 wk	12 wk
DAA schedule (cirrhotic)	Sofosbuvir/Ledipasvir	Sofosbuvir/Velpatasvir	Glecaprevir/Pibrentasvir	Sofosbuvir/Ledipasvir	Sofosbuvir/Ledipasvir
Duration	12 wk	12 wk	8-16 wk	12 wk	12 wk

DDA: Direct-acting antiviral.

chronic HEV infections[54]. Genotype 1 HEV infection can be severe in pregnancy. It has been associated with preterm labour and a maternal mortality rate of up to 30% in the third trimester[55].

The development of a HEV vaccine (Hecolin), licensed in China, has shown promise and efficacy in preventing HEV genotype 4 infection in healthy individuals 16–65 years[56].

ADVANCES

HAV

The WHO's 1992 recommendation of universal mass vaccination programmes in countries with a high risk HAV exposure has led to positive outcomes. The introduction of universal vaccination in countries with endemic HAV infections has resulted in decreased incidence of HAV as well as reduced cases amongst non-vaccinated individuals. Incidence reductions of 88% in Argentina, > 95% in Israel, 93% in Panama and 96% in Uruguay were observed over a 5-10 year period post introduction of a vaccination programme[57].

HBV

WHO elimination strategy: The WHO and its member states aim to eliminate viral hepatitis by 2030. This is a historic commitment which has allowed for funding and resource advocacy internationally. Undoubtedly the impact of the current global pandemic with COVID-19 coronavirus will adversely impact the provision of resources and funding.

Updated HBV treatment guidelines on therapy discontinuation: International guidelines have outlined endpoints for nucleos(t)ide analogues withdrawal, the optimum of which is loss of HBsAg. This is rarely achieved, however, due to the presence of covalently closed circular DNA (cccDNA). Surrogate markers of efficacy have been developed including viral suppression, transaminases normalisation and HBeAg seroconversion[12]. These markers are associated with cessation of inflammation and fibrosis advancement[58]. These surrogate markers are now considered as potential alternative endpoints: the seroconversion of HBeAg in non-cirrhotic patients with normal ALT levels and viral suppression can discontinue treatment post 1 year[12,59] (or 3 years as per APASL). Over the last year there is growing evidence of the potential benefit of stopping nucelos(t)ide analogue treatment in selected groups, including a greater reduction in quantitative HBsAg seroconversion in those who discontinue nucelos(t)ide analogue treatment[60,61].

HCV

Advent of DAAs allowing successful HCV eradication: The advent of DAAs with complete eradication of the HCV is one of the most critical advances in hepatology. Their increased efficacy, safety and tolerability make DAAs a welcome alternative to interferon-based therapy. These treatments now boast SVR rates > 90%.

Interferon-alpha (IFN-a) monotherapy was the mainstay of treatment until the 1990s. The advent of IFN-a and ribavirin combination therapy saw SVR of 38% with a 48-wk course[62]. This was further boosted to an SVR rate of 55% using pegylated-IFN[63]. Increased understanding of the HCV's molecular virology and life cycle enabled the synthesis of the first DAAs, namely the Ns3/4A protease inhibitors telaprevir and boceprevir. These, when used in combination with Peg-IFN and ribavirin, achieved an SVR rate of 65%-75%[64]. Use of this 'triple therapy' gained FDA approval in 2011. Later, sofosbuvir's development achieved 82 and 95% SVR rates when used with ribavirin alone[65]. The use of a DAA therapy alone (sofosbuvir and ledipasvir) demonstrated SVR rates of 94%-99% at 12 wk in three pivotal trials comparing DAA combinations (with and without ribavirin) in both treatment-naïve and treatment-experienced patients[66-68]. There are currently 10 FDA-approved DAAs.

HDV

Development of new treatments: Newly developed treatments for HDV are promising. These include viral assembly point inhibitors, virus entry point inhibitors (Bulvertide phase II and III trials NCT03852719, approved by EU in July 2020)[69] and prenylation inhibitors (lonafarnib in combination with ritonavir and Peg-IFN, phase III trials NCT03719313)[70].

HEV

Improved understanding of its pathogenesis and extra-hepatic manifestations: Improved understanding and awareness of extra-hepatic manifestations has improved HEV diagnosis and thus treatment. These include conditions such as Guillain-Barré[71,72], cryoglobulinemia's[73], cardiac arrythmias[74] and pancreatitis[75] to name a few.

Development of HEV vaccine in China has proved effect further study is needed to demonstrates it long-term effect[56].

CHALLENGES

HBV

Prevention of vertical transmission in African countries: The first HBV vaccine dose should be administered within 24 h of birth ("the birth dose"). This has proven challenging, particularly in countries where home births predominate. Only 94 of 194 countries have established scheduled birth dose HBV vaccination, with an estimated 38% of children worldwide receiving this crucial dose[1]. This needs to be addressed in an attempt to reduce vertical transmission.

Difficulty with early diagnosis: As previously mentioned in this article, HBV is underdiagnosed and indeed people are often diagnosed late in life. In Asia, HBV is the main cause of HCC and the main cause of liver transplantation[76]. Failure to access diagnostics and a lack of screening programmes perpetuate this situation[28].

Reactivation in the context of Immunosuppression: HBV reactivation is the reappearance or rise of HBV DNA in those with past or chronic HBV infection. Reactivation may occur in a range of clinical scenarios predominantly in the context of an immunosuppressed state of immunosuppressive therapy. Reactivation of HBV is frequently reported in patients undergoing chemotherapy for haematological malignancies and post hematopoietic stem cell transplants[77].

Chronic HBV relapse: In contrast to HCV, the current antiviral therapy does not have the potential of HBV eradication. cccDNA is a replication template of HBV: The pre-genomic portion can be reverse transcribed into DNA. This cccDNA is not targeted by antiviral nucleot(s)ide analogues and can act as a viral relapse reservoir. The uncertainty of therapy course length is associated with patient compliance, resistance, safety and financial cost. To combat therapy length uncertainty (see Advances section), EASL proposed three endpoints for antiviral treatment of chronic HBV in 2009. These are: (1) HBsAg clearance; (2) HBeAg seroconversion; and (3) Persistent inhibition of HBV DNA replication[12]. However, withdrawal of treatment is still associated with significant relapse rate and the 2015 Chinese guidelines for chronic HBV infection suggest extending therapy for three years post-HBeAg seroconversion[40]. Neither the AASLD nor the EASL advises extending therapy post-HBeAg seroconversion[12,59]. Long-term nucleot(s)ide analogue use can produce resistance and side-effects. A resistance rate of 70% has been reported in patients using lamivudine for 5-years[12]. Long term use of adefovir and tenofovir can result in renal impairment[78].

HCV

Stigma and inequity: Harm reduction strategies have been proven to work; however, the failure of implementation may be secondary to the stigma associated with persons injecting drugs. As this group tends not to vote, their concerns are not advocated. Policy makers often overlook such interventions, being swayed instead by voters' issues. Because HCV is relatively asymptomatic, a large proportion of the population are unaware of their diagnosis.

Access to DAA therapy: Global access to HBV and HCV therapy remains a problem. This is a multifactorial issue that includes access to diagnostics, therapy affordability and public health issues. Only approximately 1.7-million of the estimated 250 million individuals worldwide with chronic HBV were on treatment in 2015/2016[21]. Of the estimated 71 million people living with HCV, about 20% were aware of their diagnosis and about 5 million were treated with DAAs by the end of 2017[18]. Improved access to affordable non-hospital diagnostics is needed, as well as access to treatment. Attempts to combat these shortcomings include the addition of viral hepatitis diagnostics to the WHO's proposed Essential Diagnostics List in 2016[79]. Development of point of care molecular testing is promising, but this needs to be affordable if it is to be used in low to middle-income countries[80]. Intellectual property or patenting remains a barrier to access to generic therapy and thus, to affordable treatment. Voluntary licencing agreements and those created through the Medicines Patent Pool (MPP) allow the manufacture and sale of generic DAAs, improving affordability. The proposed licensing of pibrentasvir and glecaprevir with the MPP would facilitate access to generic alternatives of important pan-genotypic

therapy for HCV in low to middle-income countries[81]. Licensing of tenofovir and its generics with the MPP has allowed improved access in low to middle-income countries[82]. Further work and support of HCV eradication, and of HBV viral suppression programmes, are needed at both national and global level.

DAA resistance: The emergence of resistance to DAAs is a crucial challenge. The HCV high replication rate (estimated at 10^{12} copies per day) and the viral protein responsible for replications (RNA-dependent RNA polymerase, RdRp) demonstrates an absence of proofreading; therefore, HCV replication has a high error rate[83]. This results in multiple mutated viruses known as quasi-species, circulating in the blood[84]. Although the mutated viruses/quasi-species have less fitness (*i.e.*, ability to replicate) when an individual is treated with DAAs, one sees the quantity of the wildtype virus fall and the mutated virus rise. These mutated viruses/quasi-species can lead to structural changes in the protein/enzyme that the DAAs act on, thus generating resistance[85]. Resistance-associated-substitutions or RASs confer this resistance. The viral population that carry RASs are known as resistant variant (RAV). This can lead to RAV outgrowing the DAA-sensitive viruses during treatment (known as 'breakthrough') or after treatment (known as 'relapse'). Therefore, the emergence of resistance is also determined by the level of previous therapeutic drug exposure.

HDV: Arguably HDV represents the largest challenge in treatment as it confers the most severe acute and chronic hepatitis and thus worst outcomes.

CONCLUSION

In conclusion, viral hepatitis is due to a group of pathogens that are associated with significant disease complications and morbidity. The increased awareness of viral hepatitis disease burden and the arrival of effective treatments for HBV and HCV have enhanced efforts and calls for action to eradicate viral hepatitis, as outlined by the United Nations WHO 2030 agenda[17,86,87].

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 April 28; 9(2): 153-163

DOI: 10.13105/wjma.v9.i2.153

ISSN 2308-3840 (online)

MINIREVIEWS

Metabolic and biological changes in children with obesity and diabetes

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Author contributions: Matsumoto S and Nakamura T contributed equally to this work; Matsumoto S, Nakamura T, Nagamatsu F, Kido J, Sakamoto R and Nakamura K designed the research study; Matsumoto S, Nakamura T and Nagamatsu F performed the searching of the literature independently; Matsumoto S and Nagamatsu F wrote the manuscript; All authors have read and approve the final manuscript.

Supported by Japan Society for the Promotion of Science, No. JP15K09625 (in part); and The International Council on Amino Acid Science Japan Research Funding, No. ICAASJ001.

Conflict-of-interest statement: The authors declare no conflicts of interest associated with this manuscript.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in

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Abstract

The World Health Organization has stated that obesity in childhood is one of the most serious public health challenges of the 21st century. Overweightness and obesity in early childhood lead to a higher risk of overweightness and obesity in adulthood, thus conferring an increased risk of chronic inflammatory conditions, including type 2 diabetes mellitus, cardiovascular diseases, non-alcoholic fatty liver disease, and some cancers. Therefore, metabolome analysis, targeted at screening and intervening in childhood obesity, is very important. Recent studies have indicated that amino acid and lipid metabolism could influence metabolic pathways in children with obesity. For this review, we searched clinical data addressing metabolomic profiles and insulin resistance (IR) in children with obesity from inception to February 2021 in Medline, Web of Science, and Scopus. According to our search, branched-chain amino acids (BCAAs), aromatic amino acids, and acylcarnitines have reportedly been associated with IR as biomarkers for diabetes in children. BCAAs, tyrosine, and phenylalanine could be predictors of the future development of diabetes in nondiabetic subjects. In addition, it is well known that insulin regulates BCAA metabolism, and BCAA is a biomarker for IR. To interpret the mechanism behind metabolic changes in obesity, it is very important to understand the pathways and combinations related with amino acid, lipid and glucose metabolism. In this review, we summarize studies on metabolic changes to understand metabolomics in children with obesity.

Key Words: Children with obesity; Diabetes mellitus; Insulin resistance; Amino acids; Acylcarnitine; Metabolomics

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Manuscript source: Invited manuscript

Specialty type: Pediatrics

Country/Territory of origin: Japan

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): D, D
Grade E (Poor): 0

Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: February 25, 2021
Revised: March 23, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Balbaa ME, Correa-Burrows P, Gupta MK, Mrzljak A, Saha S, Zhao CF
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Ma YJ

Core Tip: Metabolome analysis is an important tool for understanding and predicting future risks of diabetes mellitus. There is some reliable literature on adults; however, that concerning children is limited due to a lack of understanding regarding metabolism in growing children. Here, we explain and discuss the biological/metabolic features in growing children and overgrown children, due to an enhanced understanding of the results of metabolome analysis.

Citation: Matsumoto S, Nakamura T, Nagamatsu F, Kido J, Sakamoto R, Nakamura K. Metabolic and biological changes in children with obesity and diabetes. *World J Meta-Anal* 2021; 9(2): 153-163
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/153.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.153

INTRODUCTION

The number of overweight children under the age of 5 years in 2016 was estimated to be over 41 million worldwide. Symptomatic pediatric lifestyle-related diseases are present in 5%-15% of children with obesity and increase at onset of adolescence, after the late elementary grade. Although there are many published mechanisms underlying the metabolic profile, those of children with obesity remain only partially understood. Obesity in childhood is a risk factor for hyperlipidemia, hypertension, type 2 diabetes mellitus (T2DM), and cardiovascular disorders in early adulthood. Moreover, severely overweight children develop pediatric lifestyle diseases, such as hyperlipidemia, diabetes, and fatty liver[1,2]. Between 1980 and 2014, the number of adults with diabetes increased from 108 million to 422 million[3], with T2DM accounting for > 90% of these cases[4]. Early detection and intervention of obesity in childhood is important to prevent both pediatric and adult lifestyle diseases[5].

Recently, investigations have been conducted on early screening and diagnosis using metabolomics, which avails much information and indications. About 141 reliable articles related to metabolomics in obesity have been published[6]. Samples were collected from blood (serum and plasma) and urine. Detection methods varied, including liquid chromatography tandem-mass spectrometry, gas chromatography tandem mass spectrometry, time-of-flight mass spectrometry, and nuclear magnetic resonance spectroscopy. Detected metabolites, which are related to T2DM and insulin resistance (IR), included amino acids (upregulated: isoleucine, alanine, proline, glutamate, palmitic acid, 2-aminoadipic acid, and lysine; downregulated: glycine, serine, and citrulline), lipids (palmitic acid), and others (propionyl carnitine, carnitine, and 2-aminoadipic acid). Evidently, all metabolites are related to each other in a complex manner; however, the core factor seems to be amino acids, according to the literature. In this review, we focus on amino acid metabolism in obesity and explain important mechanisms that have been established to date.

SYSTEMATIC REVIEW OF METABOLOMICS IN PEDIATRIC OBESITY

We searched clinical data addressing metabolomic profiles and IR in children with obesity from inception to February 2021 in Medline, Web of Science, and Scopus. The search strategy was based on medical subject headings terms. Variant combinations of the following terms were applied during the search: "childhood obesity," "obese children," "pediatric obesity," "IR," "metabolomics," "amino acids," and "lipids". Inclusion and exclusion criteria have been described in a previous study (39 Using Metabolomic Profiles as Biomarkers for Insulin Resistance in Childhood Obesity: A Systematic Review). QUADOMICS was used to evaluate the methodological quality of the included studies. We performed literature selection according to the PRISMA protocol shown in Figure 1[7]. In this review, we summarize not only systematically reviewed data but also basic studies that help in understanding metabolic changes in children with obesity. We were able to detect 13 reliable clinical studies after systematic selection (Table 1).

Table 1 Characteristics of study findings and identified metabolomic profiles in 13 clinical studies

No.	Ref.	Major finding
1	Perng *et al*[36], 2020	BCAA levels and products of BCAA catabolism were higher in males than females with comparable BMI z-score; In multivariate analyses, HOMA-IR in males correlated positively with BMI z-score and a metabolic signature containing BCAA, uric acid, and long-chain acylcarnitines and negatively with byproducts of complete fatty acid oxidation
2	Hosking *et al*[14], 2019	In longitudinal analysis, IR was associated with reduced concentrations of BCAA, 2-ketobutyrate, citrate and 3-hydroxybutyrate, and higher concentrations of lactate and alanine
3	Suzuki *et al*[44], 2019	HOMA-IR was positively correlated with valine, leucine, isoleucine, phenylalanine, tryptophan, methionine, threonine, lysine, alanine, tyrosine, glutamate, proline, arginine, ornithine, total free amino acids and aspartate; Blood uric acid levels were positively correlated with leucine and glutamate, and negatively correlated with serine, glycine, and asparagine
4	Perng *et al*[34], 2018	BCAA and androgen hormone metabolite patterns are related to changes in metabolic parameters in a sex-specific manner during early adolescence
5	Xia *et al*[37], 2018	Disrupted arginine and proline metabolism associated with phthalate exposure might contribute to the development of overweight and obesity in school-age children
6	Moran-Ramos *et al*[38], 2017	Principal component analysis showed a serum amino acid signature composed of arginine, leucine/isoleucine, phenylalanine, tyrosine, valine and proline significantly associated with obesity and serum triglycerides
7	Goffredo *et al*[39], 2017	A branched-chain amino acid-related metabolic signature characterizes obese adolescents with non-alcoholic fatty liver disease
8	Hellmuth *et al*[40], 2016	Tyrosine alterations in association with insulin resistance precede alteration in BCAA metabolism
9	Mastrangelo *et al*[43], 2016	The majority of metabolites differing between groups were lysophospholipids (15) and amino acids (17), indicating inflammation and central carbon metabolism as the most altered processes in impaired insulin signaling
10	Lee *et al*[41], 2015	Obese children presented significantly higher levels of BCAAs and several acylcarnitines and lower levels of acyl-alkyl phosphatidylcholines; Baseline BCAAs were significantly positively correlated with both HOMA-IR and continuous metabolic risk score at the 2-year follow-up
11	Butte *et al*[42], 2015	BCAAs and their catabolites, propionylcarnitine and butyrylcarnitine, were significantly elevated in obese children; Lower lysolipids and dicarboxylated fatty acids were seen in obese children; Steroid derivatives were markedly higher in obese children, as were markers of inflammation and oxidative stress
12	Perng W *et al*[33], 2014	BCAA and androgen metabolites were associated with adiposity and cardiometabolic risk during mid-childhood
13	Newbern *et al*[35], 2014	BCAA levels and byproducts of BCAA catabolism are higher in obese teenage boys than girls of comparable BMI z-score; A metabolic signature comprising BCAA and uric acid correlates positively with HOMA-IR in males and TG to HDL ratio in females and inversely with adiponectin in males but not females

BCAA: Branched-chain amino acid; BMI: Body mass index; HDL: High-density lipoprotein; HOMA: Homeostatic model assessment; IR: Insulin resistance; TG: Triglycerides.

AMINO ACID AND INSULIN PROFILES DURING CHILDHOOD IN HEALTHY CHILDREN

In healthy children, total amino acid concentration tends to increase up to 18 years of age due to the growth and construction of their own bodies. Plasma amino acid concentrations during childhood are summarized in Table 2[8]. Nine amino acids, namely, alanine, arginine, asparagine, methionine, ornithine, phenylalanine, proline, threonine, and tyrosine, decrease during the first year after birth. After 1 year of age, the concentrations tend to increase throughout childhood and adolescence. Nine other amino acids, namely, cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, tryptophan, and valine, demonstrate a steady increase throughout infancy, childhood, and adolescence. Five amino acids, namely, aspartic acid, citrulline, glutamic acid, serine, and taurine, show a steady concentration throughout childhood. In children with obesity and without IR, total plasma free amino acids demonstrate lower levels than those in healthy controls.

Insulin exhibits change similar to those of total free amino acids, and puberty strongly influences IR, as described in Table 3[9]. During puberty, insulin increase correlates with prepubertal levels. The lower insulin sensitivity observed in prepubertal girls is likely due to a higher growth hormone secretion in pubertal girls or differences[10,11] in insulin peripheral action in both boys and girls[12]. Particularly in boys during puberty, it has been demonstrated that IR and cardiovascular risk increase[13]. Serum insulin increases in association with age, body mass index (BMI), PS, IGF-1 level, and triglyceride (TG) level[9]. In addition, Hosking *et al*[14] found IR to

Table 2 Plasma amino acid values (μmol/L) during childhood

Amino acid	6 mo 50th (10th-90th) quantile value	2 yr 50th (10th-90th) quantile value	6 yr 50th (10th-90th) quantile value	16 yr 50th (10th-90th) quantile value
Alanine	248 (182-396)	229 (173-349)	234 (182-319)	370 (240-482)
Arginine	72 (43-120)	70 (46-90)	76 (50-99)	96 (68-128)
Asparagine	41 (31-56)	38 (29-56)	42 (31-67)	55 (37-81)
Aspartic acid	7 (4-18)	6 (3-8)	4 (3-6)	4 (2-5)
Citrulline	26 (14-32)	28 (17-35)	30 (23-37)	30 (23-39)
Cystine	38 (21-53)	41 (27-52)	44 (33-54)	48 (36-61)
Glutamic acid	56 (31-113)	52 (25-81)	39 (13-65)	25 (11-46)
Glutamine	583 (474-737)	580 (473-692)	604 (493-724)	680 (551-797)
Glycine	207 (138-276)	207 (138-276)	213 (144-282)	252 (183-322)
Histidine	76 (61-91)	76 (61-91)	78 (63-93)	92 (77-107)
Isoleucine	53 (39-76)	55 (4-78)	54 (40-69)	60 (47-74)
Leucine	115 (77-153)	111 (79-147)	110 (86-136)	128 (101-159)
Lysine	129 (87-171)	130 (88-172)	139 (96-181)	200 (157-242)
Methionine	55 (25-103)	19 (13-22)	20 (14-25)	26 (20-34)
Ornithine	53 (38-78)	35 (24-60)	37 (25-50)	47 (37-62)
Phenylalanine	52 (38-78)	49 (39-65)	49 (40-61)	57 (47-74)
Proline	93 (151-265)	128 (93-220)	127 (93-201)	184 (113-271)
Serine	126 (98-160)	121 (97-154)	118 (96-155)	130 (101-177)
Taurine	59 (39-111)	55 (39-80)	53 (41-69)	53 (41-66)
Threonine	102 (61-162)	91 (61-115)	94 (65-125)	131 (104-188)
Tryptophan	54 (34-73)	54 (35-73)	56 (37-76)	74 (54-87)
Tyrosine	60 (43-108)	55 (40-77)	55 (39-65)	65 (46-87)
Valine	196 (135-260)	199 (147-255)	199 (165-234)	233 (178-275)

Table 3 Insulin, glucose/insulin, and homeostasis model assessment of insulin resistance levels during childhood

Group	Insulin	Glucose/insulin	HOMA-IR
Prepubertal			
≤ 7.5 yr	2.9 (1.6-10.9)	29 (12-50)	0.6 (0.3-1.4)
> 7.5 yr	5 (1.7-9.6)	20 (10-47)	1.1 (0.3-2.0)
Pubertal			
Girls	6.7 (1.8-16.9)	12 (7.9-44)	1.6 (0.3-2.6)
Boys	7.3 (2.2-12.9)	16 (8.0-44)	1.1 (0.3-2.4)

G/Ins: Glycemia/insulin ratio; HOMA-IR: Homeostasis model assessment of insulin resistance; Insulin level: μIU/mL: G/Ins ratio: G (mg/dL) and Ins (μIU/mL); HOMA-IR: Ins (μIU/mL) × G (mmol/L)/22.5.

be associated with lower concentrations of branched-chain amino acids (BCAAs), 2-ketobutyrate, citrate, and 3-hydroxybutyrate and higher concentrations of lactate and alanine. These data portray the biochemical consequences of IR as rapid metabolism, ketogenesis, and pyruvate oxidation during normal child growth and development. Hence, when children undergo metabolic analysis, these factors, which potentially influence metabolism in the body, should be considered thoroughly and matched sufficiently in evaluating the results.

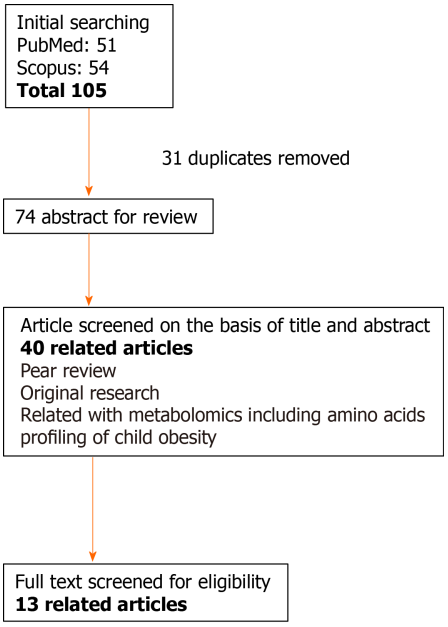


Figure 1 Flow diagram of the literature search and study selection for "metabolic/biological change in children with obesity and diabetes".

INSULIN REGULATES GLUCOSE METABOLISM AND MODIFIES AMINO ACID METABOLISM

Insulin regulates glucose homeostasis mainly in the liver, muscle, and fat. Insulin inhibits gluconeogenesis and glycogenolysis, leading to induced glycogen storage[15]. Insulin promotes the uptake, storage, and use of glucose in muscle and fat. Insulin is responsible for the induction of potassium transport in muscle, differentiation into adipocytes[16], production of androgens by ovaries[17], and retention of sodium by the kidney[18]. Insulin performs all these functions by binding with a specific tyrosine kinase-type of transmembrane receptor. The binding of insulin to receptors leads to signal transduction, including insulin-receptor substrate-1 and PI3K, which activates Akt. Akt phosphorylates the Ser9 site of GSK-3b and inhibits its activity, leading to glycogen synthesis (Figure 2).

In addition, insulin, which acts as an inhibitor of protein degradation, reduces essential amino acid concentrations in the post-absorptive state. Insulin infusion leads to hypoaminoacidemia and does not stimulate protein synthesis in skeletal muscle or splanchnic tissues during the post-absorptive state[19]. Insulin infusion decreases muscle protein degradation and does not enhance protein synthesis without amino acid intake[10,20,21]. In growing children without IR, total plasma free amino acid levels are lower than those of adults (Table 3).

METABOLIC CHANGE IN PREDIABETES AND T2DM

Metabolic change in adults is summarized in Figure 3. BCAAs are known to be associated with obesity, a phenomenon first reported by Felig *et al*[22] in 1969. The plasma concentrations of BCAAs and aromatic amino acids (ArAAs), such as Phe and Tyr, were increased and that of glycine was decreased in individuals with obesity when compared to those of age- and sex-matched healthy controls. Consequently, Newgard *et al*[23] reported in 2009 that principal component analysis performed on datasets comprised plasma and urine amino acids, acylcarnitines, organic acids, and fatty acids. They identified a cluster of metabolites comprising BCAAs, ArAAs (Phe and Tyr), Glu/Gln, Met, and C3 and C5 acylcarnitines that are strongly associated with IR, suggesting a fundamental alteration of BCAA metabolism in insulin-resistant states. On the other hand, glycine was negatively associated with IR when measured using the homeostatic model assessment (HOMA) score[24] or by hyperinsulinemic-euglycemic clamp[20]. In patients with T2DM, the concentrations of BCAAs can be up to 1.5-fold, or even 2-fold, higher than those in healthy subjects, with a mean change

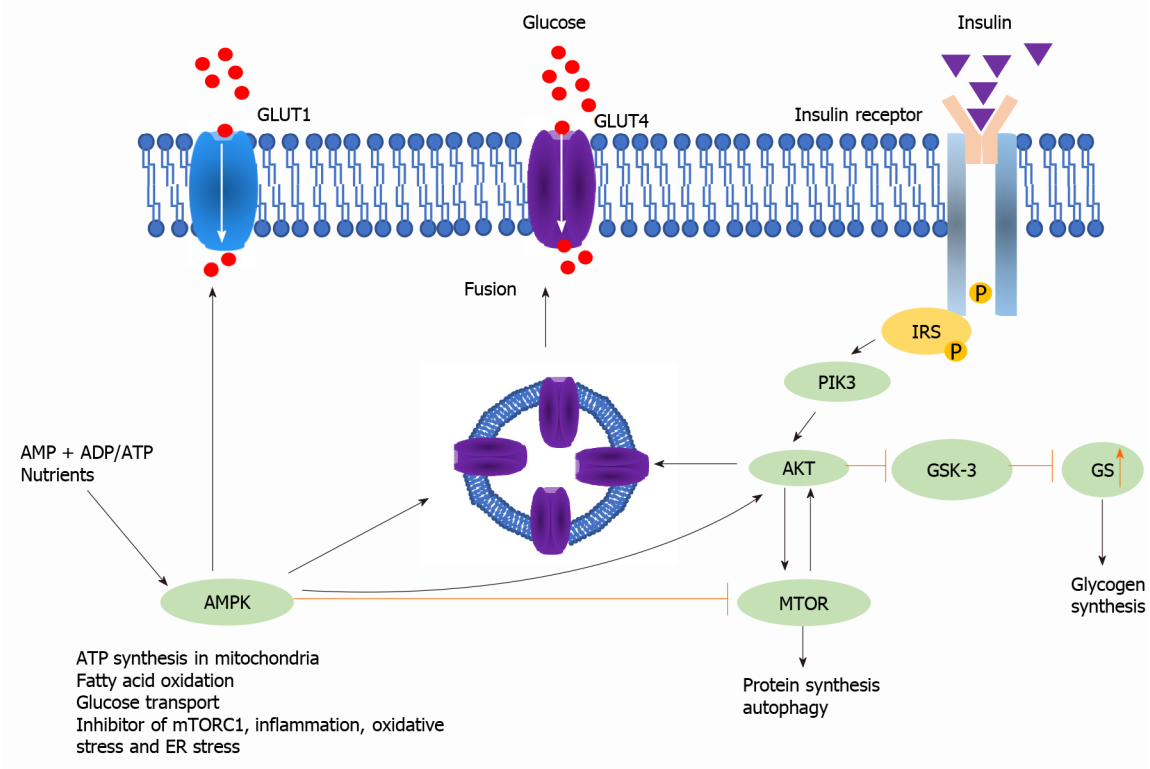


Figure 2 Insulin signaling pathway. After insulin binds to the insulin receptor, which is a type of tyrosine kinase, signal transduction mediated by insulin-receptor substrate-1, PI3K, and AKT is activated by phosphorylation. AKT phosphorylates the Ser9 site of GSK-3b, which inhibits glycogen synthetase (GS). Hence, the insulin signaling pathway leads to glycogen synthesis. AMPK and MTOR are major modulators in metabolism. Insulin resistance and branched-chain amino acids are indicated to influence these mediators.

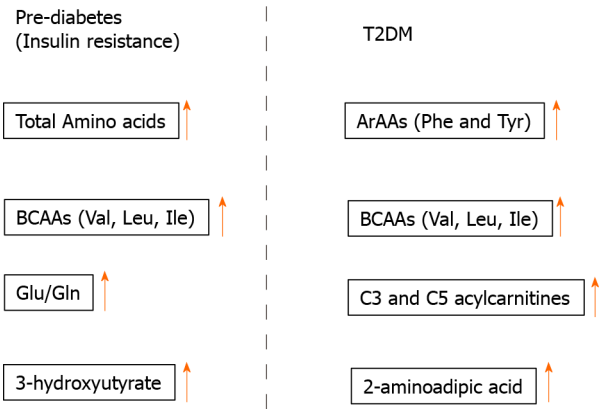


Figure 3 Insulin signaling pathway. BCAAs: Branched-chain amino acids; T2DM: Type 2 diabetes mellitus.

below 1.5-fold (1.2-1.3). These results are reproducible and statistically significant[25-27].

The strong association of BCAAs with IR has been reported in several studies, including a cross-sectional study of subjects with metabolic syndrome and varying BMI[28], a study on Chinese and Asian-Indian subjects residing in Singapore in which BMI was controlled (average BMI of 24)[29], a study on subjects at extremes of insulin sensitivity in the Insulin Resistance Atherosclerosis Study[30], and studies on the effects of combined aerobic and resistance training in insulin-resistant subjects[31]. In all of these studies, the BCAA-related factor was more correlated with IR than any lipid-related factor.

Another candidate metabolite is 2-aminoadipic acid (2-AAA), which is strongly correlated with incident T2DM, with subjects in the top quartile for this metabolite having a > 4-fold increase risk of disease[32]. 2-AAA proved to be an independent biomarker because its levels were not associated with BCAAs or ArAAs in this study.

The association between cardiometabolic diseases and certain lipid metabolites commonly measured in clinical chemistry laboratories - including TGs, cholesterol, and total nonesterified fatty acids - has long been recognized, leading some to conclude that cardiometabolic diseases are driven by perturbed lipid homeostasis. Application of metabolomics has led to a broader appreciation of metabolites that associate with these maladies and, in some cases, metabolites that predict intervention outcomes and future disease development.

METABOLIC CHANGE IN CHILDREN WITH OBESITY AND IR

Perng *et al*[33] investigated plasma metabolic profiling in 262 children (6-10 years of age). They found that BCAA-related and androgen-hormone patterns were higher in obese than in lean children. Both patterns were associated with adiposity and worse cardiometabolic profiles. Each increment in the BCAA and androgen pattern scores corresponded with a 6% (95% confidence interval (CI): 1%-13%) higher HOMA-IR. Children of mothers with obesity had higher BCAA scores than in those of mothers without obesity. Perng *et al*[34] also reported a BCAA pattern corresponding with a 4.82 (95%CI: 0.92-8.71) mg/dL decrease in fasting glucose in boys. In girls, the BCAA pattern was associated with an increase in TGs [4.17 (0.03-8.32) mg/dL]. The androgen pattern was associated with decreased leptin [-2.35 (-4.34 to -0.35) ng/dL] and increased C-reactive protein [0.28 (0.03-0.54) mg/dL] in girls. These relationships did not differ by baseline BMI percentile. Sex differences in biomarkers were reported by Newbern *et al*[35]. They investigated metabolome profiles in 82 adolescents with obesity. BCAA levels and byproducts of BCAA catabolism were higher in teenage boys with obesity than in girls of comparable BMI z-scores. A metabolic signature comprising BCAA and uric acid correlates positively with HOMA-IR in males and TG-to-high-density lipoprotein ratio in females and inversely with adiponectin in males but not females.

Perng *et al*[36] also investigated metabolomic profiles of four overweight/obesity and metabolic risk phenotypes among 524 adolescents aged approximately 13 years. Their data demonstrated that long-chain fatty acids, BCAAs, acylcarnitines, diacylglycerols, and steroid hormones differed by weight status and metabolic phenotype. Xia *et al*[37] investigated urinary metabolic analysis in 69 children with overweight/obesity and 80 lean children. They discovered that mono-n-butyl phthalate (MnBP) exposure was significantly associated with overweight/obesity in children and the disrupted arginine and proline metabolism might have played an important role in mediating the putative obesogenesis of MnBP. Moran-Ramos *et al*[38] investigated data from the Obesity Research Study for Mexican Children conducted in Mexico City and included a case control (n = 1120), cross-sectional (n = 554), and longitudinal study (n = 301) of 6-12-year-old children. Principal component analysis presented a serum amino acid profile composed of arginine, leucine/isoleucine, phenylalanine, tyrosine, valine, and proline significantly associated with obesity [odds ratio (OR) = 1.57; 95%CI: 1.45-1.69, P = 3.84 × 10-31) and serum TGs (β = 0.067, P = 4.5 × 10-21). In the longitudinal cohort, the amino acid profile was associated with serum TG and the risk of hypertriglyceridemia after 2 years (OR = 1.19; 95%CI: 1.03–1.39, P = 0.016). They found that an amino acid profile is an independent risk factor for future hypertriglyceridemia in childhood. Goffredo *et al*[39] investigated 180 metabolites in 78 adolescents with obesity with (n = 30) or without (n = 48) non-alcoholic fatty liver disease (NAFLD) assessed by magnetic resonance imaging. Adolescents with NAFLD had higher plasma levels of valine (P = 0.02), isoleucine (P = 0.03), tryptophan (P = 0.02), and lysine (P = 0.02) after adjustment for confounding factors. BCAAs were negatively correlated with peripheral and hepatic insulin sensitivity. Higher baseline valine levels predicted an increase in hepatic fat content at follow-up (P = 0.01). Hellmuth *et al*[40] studied 80 children with obesity before and after a 1-year lifestyle intervention program. Only tyrosine was significantly associated with HOMA (P < 0.05) at baseline and end, and with change during the intervention (P < 0.05). Lee *et al*[41] analyzed 186 plasma metabolites in 109 Korean boys at baseline and after 2 years. Significantly higher levels of BCAAs and several acylcarnitines were observed in children with obesity and as well as lower levels of acyl-alkyl phosphatidylcholines. Baseline BCAAs were significantly positively correlated with both HOMA-IR and continuous metabolic-risk score at the 2-year follow-up. Baseline BCAA concentration was identified as a predictor of future risk of IR and metabolic syndrome. Butte *et al*[42] analyzed the metabolic profiles of 353 Hispanic children without obesity and 450 with obesity. BCAAs, BCAA-catabolites, propionylcarnitine, and butyrylcarnitine

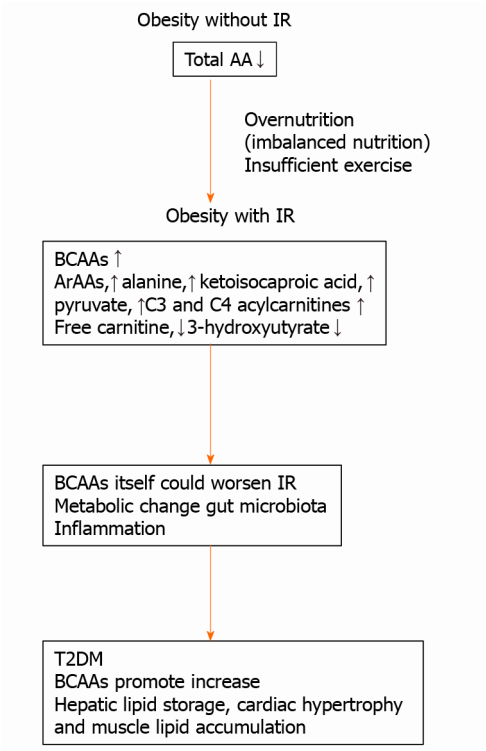


Figure 4 Metabolic change in children with obesity. BCAAs: Branched-chain amino acids; T2DM: Type 2 diabetes mellitus.

were significantly elevated in children with obesity.

Mastrangelo *et al*[43] reported in 2016 on the metabolic profiles of prepubertal children with obesity. They found that BCAAs, ArAAs, alanine, ketoisocaproic acid, pyruvate, and C3 and C4 acylcarnitines increased in children both with obesity and IR (Figure 4). In addition, free carnitine and 3-hydroxyutyrate decreased in children presenting obesity with IR. Because ArAAs potentially compete with BCAAs for transport into mammalian cells, their elevation in serum may indicate a disruption in their transportation into the brain, leading to an excess of BCAAs. Suzuki *et al*[44] reported in 2019 about relationship among amino acids, lipids, and glucose metabolism. In this study, HOMA-IR was positively correlated with BCAAs, ArAAs, Ala, and Glu/Gln, with statistical significance[28]. The mechanism underlying this profiling has not been determined; however, a hypothesis has been suggested. Pozefsky *et al*[45] suggested that impaired insulin activity and decreased utilization of amino acids in the muscles increased plasma BCAA levels owing to reduced uptake of BCAA in the muscles in lifestyle-related diseases. Newgard[46] reasoned that the increased circulating blood BCAA in obese and insulin-resistant subjects partly results from a decline in amino acid (AA) catabolism in their adipose tissue. Readily usable glucose and lipid substrates are considered to obviate the need for AA catabolism in adipose tissue by downregulation of the BCAA catabolic enzymes through the suppression of peroxisome proliferator-activated receptor-γ signaling in such metabolic adaptations. Würtz *et al*[47] reported that gluconeogenesis substrates, including Ala, increased in adults with impaired glucose tolerance. Moreover, Shimizu *et al*[48] reported that depletion of plasma Ala serves as a cue to increase plasma fibroblast growth factor 21 values and enhance liver-fat communication, resulting in the activation of lipolytic genes in adipose tissues. Increases in circulating BCAAs in obesity result in part from decreased rates of their oxidation in adipose tissue, due to coordinated transcriptional suppression of all the BCAA catabolic enzymes as well as from increased phosphorylation and inactivation of the branched-chain ketoacid dehydrogenase (BCKDH) complex in the liver, such that fewer BCAAs are taken up from the blood. Rodent models of obesity exhibit increased hepatic expression of the inhibitory BCKDH kinase, BDK, and reduced expression of the activating BCKDH phosphatase, PPM1K[49]. Transplantation of microbiota from humans with obesity into germ-free mice causes a significant increase in circulating BCAAs and obesity[50]. Hence, gut microbiota is a candidate donor of BCAAs. BCAA biosynthetic pathways are induced in the gut microbiota of obese compared to that of lean humans.

CONCLUSION

Obesity leading to several complications during adulthood is a critical issue to be resolved and protected against during childhood. Metabolomics is a common tool for investigating not only adult but also children with obesity, whose data potentially provide clear fundamental mechanisms and evaluations. Nutrient factors, such as glucose, amino acids, and lipids, influence and relate with each other; nevertheless, amino acids are a critical modifier in obesity. Because amino acids reflect the internal metabolic change and intestinal microbiota and act as metabolic modulators, monitoring of amino acids by metabolome analysis may be a useful method to predict metabolic disorders during childhood.

ACKNOWLEDGEMENTS

We thank all the staff at our institution who cooperated in the collection of patient samples as well as those at the Department of Central Clinical Laboratory, Kumamoto University Hospital.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 April 28; 9(2): 164-175

DOI: 10.13105/wjma.v9.i2.164

ISSN 2308-3840 (online)

MINIREVIEWS

Nonalcoholic fatty liver disease and cardiovascular concerns: The time for hepatologist and cardiologist close collaboration

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Author contributions: Ashraf H and Jamali R proposed the idea; Ashraf H, Karbalai S and Jamali R searched the database and extracted the articles; Ashraf H and Jamali R wrote the manuscript; All the authors read and approved the final draft.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has emerged as the most common chronic liver cell damage worldwide. It is strongly associated with an increased risk of cardiovascular disease (CVD). There are not enough recommendations for screening subjects with nonalcoholic steatohepatitis cirrhosis, who are not candidates for liver transplantation, nor who are asymptomatic with NAFLD without cirrhosis. In the current comprehensive narrative review, we aimed to evaluate the associations between CVD and NAFLD. Distinguishing the mechanisms linking these two disorders offers the opportunity to develop targeted therapies. Moreover, we will discuss screening approaches (whom and how-to) and treatment modalities proposed to reduce cardiovascular risk in patients with NAFLD.

Key Words: Nonalcoholic steatohepatitis; Cardiovascular disease; Screening; Atherosclerosis; Risk scores; Metabolic syndrome

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Core Tip: The diagnosis of nonalcoholic fatty liver disease (NAFLD) deserves a thoughtful cardiovascular risk assessment and evaluation for subclinical atherosclerosis to prevent cardiovascular disease (CVD) morbidity/mortality. In patients with nonalcoholic steatohepatitis-related cirrhosis undergoing liver transplantation, screening for significant CAD should be done by stress echocardiography and computed tomography coronary angiography. Recommendation for screening in asymptomatic NAFLD without significant fibrosis is not clear. The basis of NAFLD prevention and treatment is lifestyle modifications, while concomitant cardiovascular risk factors should be targeted.

and hepatology

Country/Territory of origin: Iran

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: January 29, 2021
Peer-review started: January 29, 2021
First decision: March 14, 2021
Revised: March 17, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Kong L, Zhou Y
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li JH

Citation: Ashraf H, Karbalai S, Jamali R. Nonalcoholic fatty liver disease and cardiovascular concerns: The time for hepatologist and cardiologist close collaboration. *World J Meta-Anal* 2021; 9(2): 164-175
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/164.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.164

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is considered as the histologic evidence of an accumulation of triglycerides (TGs) in hepatocytes in the absence of a causative factor. It encompasses a pathological spectrum, ranging from steatosis (more than 5% of hepatocytes) to nonalcoholic steatohepatitis (NASH) (fatty infiltration plus necroinflammation and fibrosis) and finally to cirrhosis[1]. NAFLD has emerged as the most common chronic liver disease worldwide. It has constantly increasing incidence and prevalence, with a global prevalence of about 25%[2]. According to a systematic review and meta-analysis in Iran, the prevalence of total, mild, moderate, and severe types of NAFLD was estimated as 33.9%, 26.7%, 7.6%, and 0.5%, respectively[3]. It is strongly associated with an increased risk of cardiovascular disease (CVD), including subclinical atherosclerosis, coronary, cerebral, and peripheral vascular disease, myocardial dysfunction, hypertrophy, and remodeling, valvular heart disease, arrhythmia, and hypertension (Figure 1). The patients show a high risk of fatal and non-fatal CVD events, which occur in 5.2% of subjects. Biopsy-confirmed NAFLD is associated with up to a 64% increased mortality risk compared to the general population, with CVD as the most frequent cause of death (25% of deaths)[4].

In the current comprehensive narrative review, we aimed to review the association between CVD and NAFLD. Distinguishing the mechanisms linking these two disorders offers the opportunity to more considerably develop targeted therapies. Thereafter, we will discuss screening approaches (whom and how-to) and treatments proposed to reduce cardiovascular risk in patients with NAFLD.

PATHOPHYSIOLOGICAL LINK BETWEEN NAFLD AND CVD

NAFLD and CVD attend both presentations of end-organ damage of metabolic syndrome. The specific contribution of NAFLD to increased CVD risk is difficult to discriminate, due to concomitant shared risk factors. Accumulation of dysfunctional visceral adipose tissue in the liver and other organs (such as the pericardium, pancreas, kidneys, or skeletal muscle), subsequent activation of inflammatory pathways, and release of fat-derived toxic metabolites lead to the development of both NAFLD and CVD. This occurs possibly *via* mechanisms beyond classic risk factors and metabolic syndrome components[5,6]. Epicardial adipose tissue (EAT) produces proatherogenic, proinflammatory, and prothrombotic adipocytokines, which due to its anatomical intimacy with the heart muscle. EAT shares an unobstructed microcirculation with the adjacent myocardium. The underlying mechanisms linking NAFLD to CVD are very complex and simultaneously involve several different pathways, which are described as follows (Figure 1).

Endothelial dysfunction

The main causes of endothelial dysfunction are increased levels of asymmetric dimethyl arginine (ADMA), hyperhomocysteinemia, and systemic inflammation. ADMA is an endogenous antagonist of nitric oxide synthase that disturbs vasomotor regulation of vascular permeability and cause platelet dysfunction. Hyperhomocysteinemia is associated with increased intrahepatic vascular resistance, which impairs nitric oxide formation, and causes oxidative stress and platelet activation. Systemic inflammation associated with NAFLD increases endothelial dysfunction, alters the vascular tone, and enhances vascular plaque formation[5,6].

Atherogenic dyslipidemia

NAFLD changes the serum lipid profiles, causing high TGs, very low-density lipoprotein (VLDL), apolipoprotein B, and low-density lipoprotein (LDL) levels, dense and smaller LDL particle size and peak diameter, and decreased high-density lipoprotein (HDL) levels. This abnormal serum lipoproteins composition is likely to

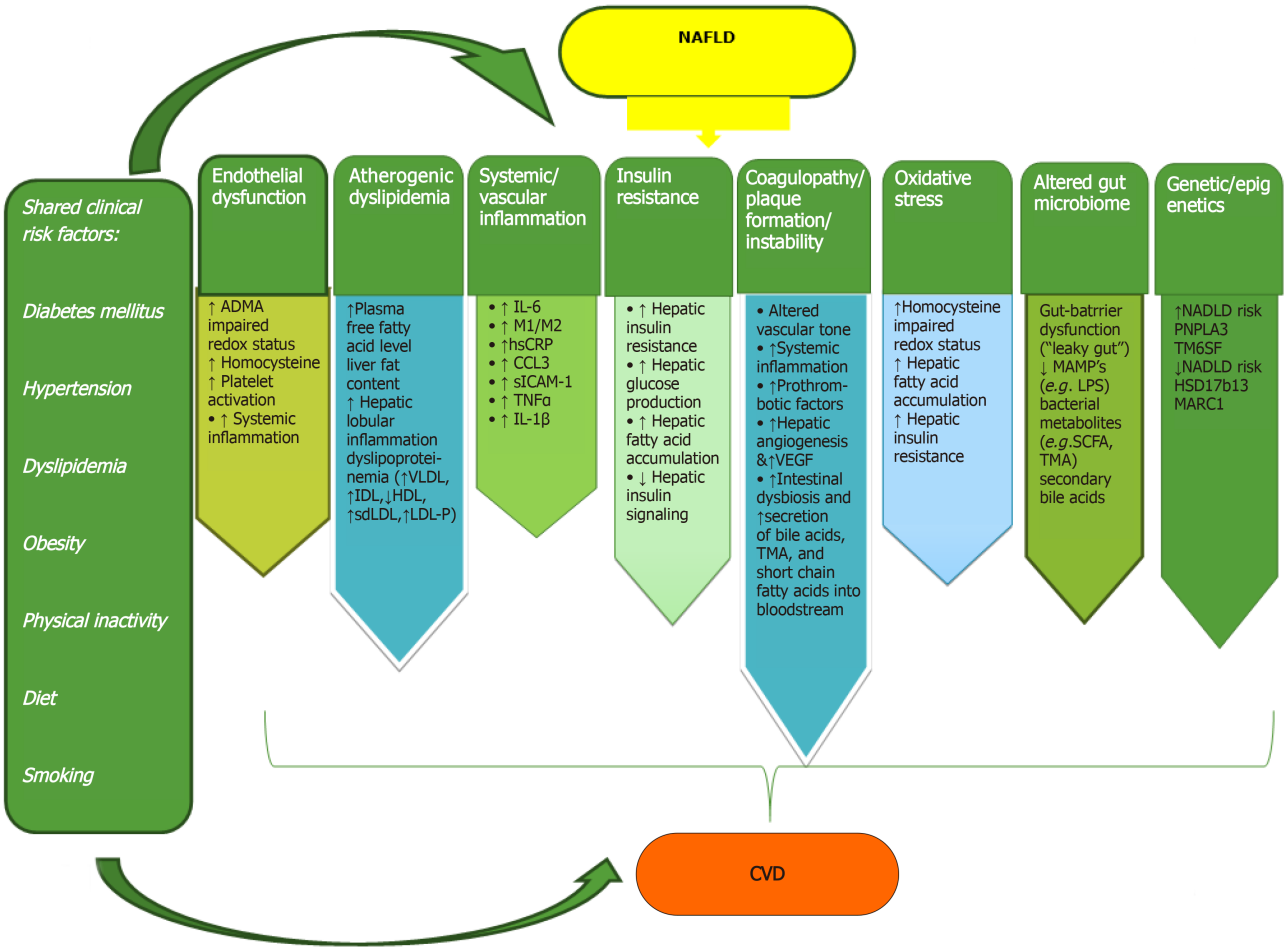


Figure 1 Schematic figure depicting potential pathways for increased risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. Down arrows indicate decreased levels, and up arrows indicate increased levels. ADMA: Asymmetric dimethylarginine; CCL3: Chemokine (C-C motif) ligand 3; CVD: Cardiovascular disease; HDL-C: High-density lipoprotein cholesterol; hsCRP: High sensitivity C-reactive protein; HSD17B13: Hydroxysteroid 17 beta-13; IDL: Intermediate-density lipoprotein; IL1b: Interleukin 1b; IL6: Interleukin 6; LDL: Low-density lipoprotein; LDL-P: Low-density lipoprotein particles; M1/M2: Macrophage phenotype 1/2 ratio; MAMP: Microbe-associated molecular pattern; MARC1: Mitochondrial amidoxime-reducing component 1; NAFLD: Nonalcoholic fatty liver disease; PNPLA3: Patatin-like phospholipase domain-containing protein 3; SCFA: Short-chain fatty acids; sdLDL: Small dense low-density lipoprotein; sICAM1: Soluble intracellular adhesion molecule 1; TM6SF: Transmembrane 6 superfamily 2 human gene; TMA: Trimethylamine; TNF-α: Tumor necrosis factor-α; VEGF: VASCULAR endothelial growth factor; VLDL: Very-low-density lipoprotein.

activate an inflammatory pathway involved in the pathogenesis of atherosclerotic CVD[7].

Systemic insulin resistance

Fatty acid accumulation in the liver, subclinical inflammation, oxidative stress, and altered adipokines leads to the suppression of endogenous liver glucose production, and further stimulating insulin resistance (both hepatic and peripheral). The mentioned mechanisms would describe the increased risk of insulin resistance with adverse cardiovascular events[8].

Proinflammatory and pro-oxidative states

Emerging evidence also suggested that serum and liver inflammatory and oxidative markers (including interleukin-6, tumor necrosis factor-α, C-reactive protein, and other proinflammatory cytokines) are increased in NAFLD. These pathways, especially in its necroinflammatory form, might be involved in the pathogenesis of cardiovascular involvement[5,6].

Coagulopathy/plaque formation/instability

Patients with NAFLD have a procoagulant imbalance that increases from the less severe (steatosis) to more severe (NASH) stages of disease. This imbalance was caused by the increased serum levels of coagulation factors (VIII, IX, XI, and XII, von Willebrand factor, fibrinogen, and plasminogen activator inhibitor type 1), while

anticoagulant factors (like antithrombin III and protein C) are decreased[9]. Another aspect that may affect plaque formation and plaque instability in NAFLD patients includes elevated serum concentrations of vascular endothelial growth factor (VEGF) and signs of active angiogenesis, indicative of vascular remodeling. However, the functional role of VEGF in atherogenesis of NAFLD patients is still unclear[9].

Genetic/epigenetics

The role of genetic factors linking NAFLD and CVD is so far unclear. Two missense genetic variants have been detected by genome-wide association reports in NAFLD: Transmembrane 6 super family member 2 (TM6SF2) and patatin-like phospholipase domain-containing protein 3 (PNPLA3). PNPLA3 regulates the physiology and morphology of lipid droplets and appears to be related to TG metabolism. Carriers of PNPLA3 have been shown to have lower serum TG levels but paradoxically increased atherosclerosis, while carriers of TM6SF2 may have reduced levels of serum TG, LDL cholesterol, and total cholesterol with subsequently experience some degree of cardioprotection[10].

Altered gut microbiome

Alterations in the gut microbiota composition, named as dysbiosis, has been identified as a contributing factor for the development of cardiometabolic diseases like NAFLD. However, a NAFLD-specific gut microbiota signature has not been detected yet. Dysbiosis increases mucosal barrier permeability to intestinal microbes and/or microbial products, designated as microbe- or pathogen-associated molecular patterns. Damage-associated molecular patterns (*e.g.*, released from damaged enterocytes), lipopolysaccharide, or peptidoglycans trigger a systemic gut dysbiosis-related inflammatory response[11].

IMPACT OF NAFLD ON CARDIOVASCULAR MORBIDITY AND MORTALITY

Whether NAFLD is an independent risk factor for cardiovascular mortality and other cardiovascular events remains controversial. Most studies demonstrate an association between NAFLD and increased CVD events, especially for those with severe NAFLD/NASH, but the association with overall or CVD mortality is less elucidated[4]. A meta-analysis containing 16 observational research studies (9 prospective and 7 retrospective cohort studies) by Targher *et al*[4] including 34,043 patients with a median 7-year follow-up showed that NAFLD patients had a 64% higher risk of having fatal or non-fatal cardiovascular events (cardiac death, myocardial infarction, need of coronary revascularizations, or stroke) as compared to patients without NAFLD (odds ratio (OR): 1.64; 95% confidence interval (CI): 1.26-2.13). However, a sub-analysis of studies with CVD mortality as the primary outcome, the association between NAFLD and fatal CVD events did not remain statistically significant.

SEVERITY OF NAFLD HISTOLOGY AND CVD MORTALITY RISK

The histologic severity of NASH was associated with the degree of carotid artery intimal medial thickness, independently of conventional cardiovascular risk factors and metabolic syndrome features. Meanwhile, the severity of NAFLD histology did not further increase CVD mortality risk[12].

CARDIOVASCULAR COMORBIDITIES IN NAFLD PATIENTS

Arterial hypertension

Systemic arterial hypertension prevalence varies from 40%-70% among NAFLD patients, and prospective epidemiological studies have demonstrated a 2-3-fold increase in the incidence of arterial hypertension over observation periods of 9 and 5 years, respectively[13,14].

Atherosclerotic CVD

Patients with NAFLD are at increased risk of subclinical atherosclerosis as measured

by increased carotid artery intima/media thickness, arterial stiffness, ankle-arm index, aortic pulse-wave velocity, coronary calcification, and endothelial dysfunction as measured by flow-mediated dilation. A meta-analysis which contained 85,395 subjects including 29,493 NAFLD subjects demonstrated higher risk of subclinical atherosclerosis in NAFLD than non-NAFLD patients (OR: 1.60; 95%CI: 1.45-1.78)[15]. In addition to subclinical atherosclerosis, patients with NAFLD have a higher risk of clinically significant atherosclerosis, and they may additionally have worse outcomes when experiencing acute events. Haddad *et al*[16] performed a meta-analysis involving 25,837 patients and reported that NAFLD subjects had a significantly higher risk of clinical coronary artery disease when compared to individuals without NAFLD (relative risk: 2.26; 95%CI: 1.04-4.92; $P < 0.001$).

Structural heart disease

In addition to functional cardiac alterations, structural heart diseases are also more common among NAFLD subjects. The increase in free fatty acids may result in myocardial steatosis, and subsequent changes in myocardial substrate metabolism, efficiency, and perfusion leading to impaired left ventricular contractility[17]. Myocardial remodeling, notably in the left ventricle, which is often accompanied by left ventricular systolic and/or diastolic dysfunction and ventricular hypertrophy is observed in NAFLD subjects[17]. The study of VanWagner *et al*[18] involving 2713 participants showed that NAFLD subjects had higher left ventricle filling pressure, lower early diastolic relaxation velocity, and worse absolute global longitudinal strain than those without NAFLD. NAFLD has also been shown to be independently associated with valvular heart disease, specifically aortic valve sclerosis and mitral annulus calcification[19]. NAFLD individuals would experience alterations in the EAT. The thickness of EAT is independently associated with NAFLD and its grades, and also increased risk for coronary calcification (EAT > 3.18 mm)[20].

Cardiac arrhythmias

There is growing evidence that NAFLD patients are at increased risk for cardiac arrhythmias, specifically ventricular arrhythmias, QTc prolongation, and atrial fibrillation independent of overlapping comorbidities and other risk factors. Although the specific mechanisms remain unclear, probable mechanisms are altered electrophysiological myocardial substrate secondary to pro-inflammatory and pro-oxidative states, insulin resistance-induced hypokalemia affecting the ventricular repolarization, myocardial structural changes, and autonomic dysfunction seen in NAFLD[17]. The associations between NAFLD and CVD comorbidities are demonstrated in Figure 2.

PREDICTORS OF CVD IN NAFLD

There is a strong association between higher gamma glutamyl transpeptidase (GGT) levels, even within the normal limit and during incident CVD events. In turn, the association of serum alanine aminotransferase (ALT) with CVD outcomes appears more controversial, exhibiting a U-shaped association with total mortality. The mechanism of the stronger association of GGT than ALT with CVD risk remains to be elucidated. GGT may be linked directly in the pathogenesis of atherosclerosis by participating in extracellular catabolism of glutathione and atherosclerotic plaque formation[21].

SCREENING FOR CVD IN PATIENTS WITH NAFLD

Patients with NAFLD can be divided into two groups regarding CVD evaluation. One group encompasses subjects with NASH cirrhosis who are candidates for liver transplantation, and another group comprises asymptomatic patients with NAFLD (without cirrhosis).

In patients with NASH cirrhosis who are candidates for liver transplantation, dobutamine stress echocardiography (DSE) is widely used, but the predicted maximum heart rate may not be achieved, which reduces its reliability; computed tomography (CT) coronary angiography needs a stable low heart rate, is less invasive than conventional angiography, and the negative predictive value is excellent. Myocardial perfusion imaging can also be used to detect myocardial ischemia, but studies have shown contradicting results. Coronary angiography is the gold standard

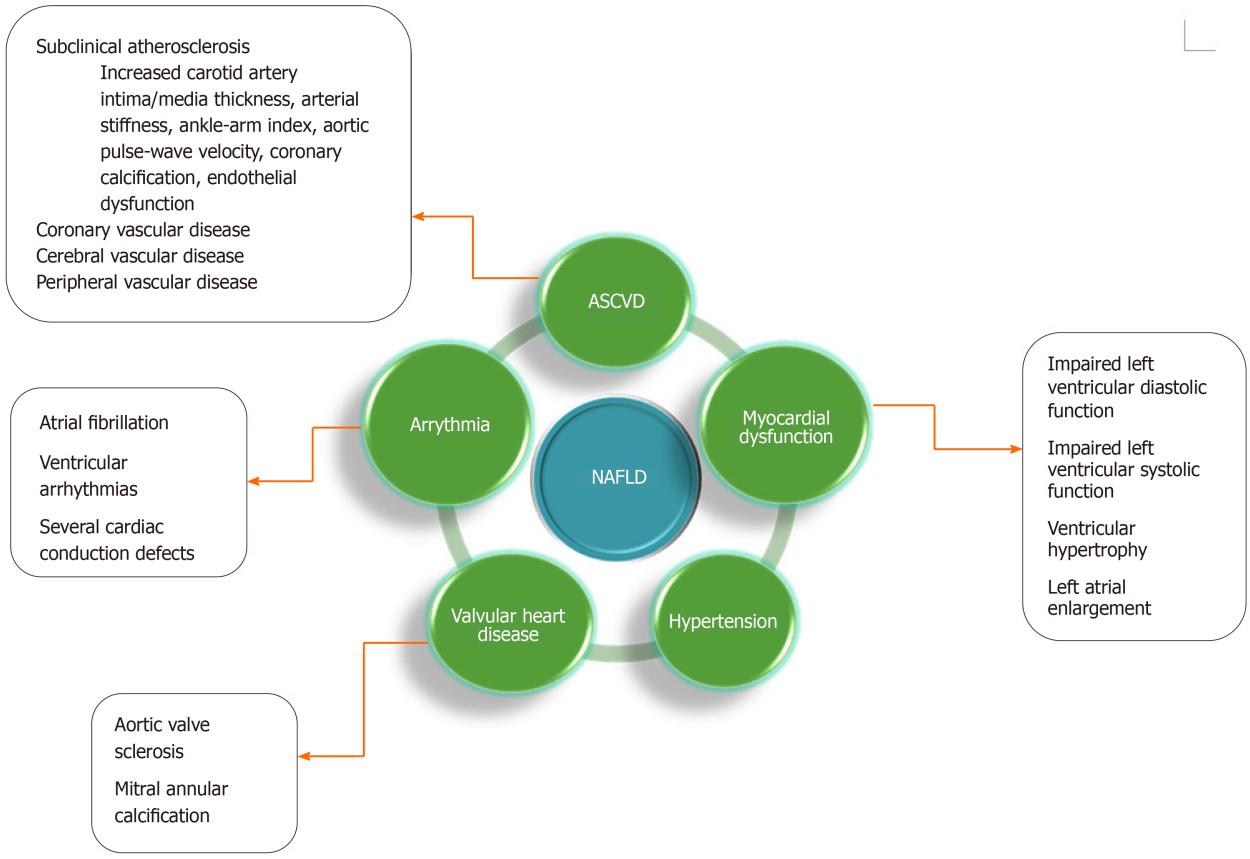


Figure 2 Nonalcoholic fatty liver disease is strongly associated with an increased risk of cardiovascular disease comorbidities. ASCVD: Atherosclerotic cardiovascular disease; NAFLD: Nonalcoholic fatty liver disease.

modality for coronary examination; however, it is an invasive test[7]. We agree with the algorithm for evaluation of CAD in pretransplant evaluation suggested by Hogan *et al*[22]. It recommended coronary angiography if more than two of the following risk factors are present: Age > 50 years, history of NAFLD, hypertension, family history of cardiovascular disease, type 2 diabetes mellitus, smoking, or known cardiovascular disease. Patients with 1 or 2 risk factors can be taken for DSE, and coronary angiography will be done for patients with abnormal DSE.

There are not enough recommendations for screening patients with NASH cirrhosis, who are not a candidate for liver transplantation, and also asymptomatic patients with NAFLD (without cirrhosis). According to the American College of Cardiology (ACC)/American Heart Association (AHA) guideline for assessment of cardiovascular risk in asymptomatic adults, once a diagnosis of NAFLD is established, either in the presence or the absence of features of the metabolic syndrome, a thorough clinical evaluation of the patient must be sought including cardiovascular family history, an assessment of physical status such as blood pressure, waist circumference, body mass index, and laboratory parameters, including fasting blood sugar, lipid profile, glomerular filtration rate, and albuminuria. For each patient, the global CVD risk must be calculated using cardiovascular risk assessment tools, among them, in particular, the Framingham risk score[23] and the ACC/AHA tool, and which have been used in patients with NAFLD are valuable to identify those at higher risk of cardiovascular events at 10 years[7]. We should keep in mind that the mentioned risk scoring systems are validated for asymptomatic individuals. Therefore, their applicability in NAFLD subjects is questionable. Meanwhile, there are some Iranian risk scores[24,25], but data in Iranian NAFLD patients is also scarce[26,27] and risk scores in these patients are not validated. The Framingham risk score is a traditional risk factor inventory-based scoring system (http://www.framinghamheartstudy.org/risk/coronary.html) that uses readily available variables, such as gender, age, cigarette use, total cholesterol, high-density lipoprotein cholesterol, and systolic blood pressure to estimate the 10-year cardiovascular risk. This score stratifies subjects as low (< 10%), intermediate (10%-20%), or high (> 20%) CVD risk. However, the Framingham risk score has several limitations; primarily, it can overestimate or underestimate risk in understudied populations. Also, it does not predict the risk for heart failure, transient ischemic

attack, or stroke[23]. Due to these limitations, in 2013, the ACC/AHA Task Force released a new risk assessment tool (http://www.cvriskcalculator.com/) to assess 10-year atherosclerotic cardiovascular disease (ASCVD) risk, called the Pooled Cohort Equation, which uses race, sex, age, total cholesterol, HDL, systolic blood pressure, smoking, treatment for hypertension and diabetes as risk factors[7].

We believe that patients with low-risk by risk scores should be managed with lifestyle modification. Patients with intermediate- or high-risk by risk scores should be referred to a cardiologist for evaluation of CVD. The question remains, however, when is the time for hepatologist and cardiologist close collaboration. Guidelines by the ACC/AHA recommend screening for subclinical atherosclerosis using coronary artery calcifications and carotid artery intima-media thickness in intermediate-risk asymptomatic individuals to modulate the intensity of treatment. Although coronary artery calcifications may have a higher prognostic value in predicting future cardiac events, cost and radiation exposure are their main concerns, and ultrasonography of the carotid artery maybe the initial screening test of choice. Table 1 summarizes methods to screen CVD in asymptomatic patients based on AHA guidelines[7]. The proposed algorithm for the screening of CVD in patients with NAFLD is provided in Figure 3.

CVD RISK MEASUREMENT INTERVALS

The timing and frequency of the reassessment of global cardiovascular risk in patients in primary prevention of CVD (including those with NAFLD) are still poorly elucidated. The physician should make decisions on the frequency of remeasuring for cardiovascular risk according to risk measured at baseline. Re-estimation of the risk before 8-10 years is not obligatory for most subjects initially not requiring therapy. However, yearly re-evaluation seems warranted in those with an initial risk of 15% to < 20%.

SCREENING FOR NAFLD IN CVD

Screening for fatty liver in atherosclerotic coronary disease patients seems to be a reasonable approach. The mortality risk is dependent on the presence and extent of fibrosis, and those with simple steatosis were not at increased risk of CVD death. If a cheap, non-invasive, available, and reliable tool to detect high-risk patients (*i.e.* NASH with some degree of fibrosis) were available, the answer to the above-mentioned question may be yes. Screening for NAFLD using ultrasound in patients with ASCVD may not be clinically possible; patients with ASCVD should be considered a high-risk group, at minimum according to accompanying risk factors. A recent study showed the relationship between NAFLD fibrosis score and cardiovascular risk[28]. It suggested that liver fibrosis biomarkers could be applied in primary care cardiovascular risk assessment.

PROPOSED THERAPIES TO REDUCE CVD

While no specific pharmacologic therapy is provided for the liver itself, optimal management of metabolic risk factors is crucial to reduce cardiovascular risk, which means killing two birds with one stone.

Nonpharmacological modalities

The gold standard treatment for NAFLD is lifestyle modification. It includes dietary change, appropriate physical activity, and weight loss approaches.

Getting to ideal body weight: Weight reduction can be achieved by caloric restriction diet, aerobic exercises, and bariatric surgery in morbid obese patients. A total weight reduction of about 5% is suggested for possible reduction in the amount of liver fat content and the improvement of metabolic abnormalities, 7%-10% for decreasing liver cell inflammation, and up to 10% for substantial fibrosis regression. However, weight loss alone is not sufficient for reducing the cardiovascular risk of NAFLD patients. It should always be integrated with further lifestyle modifications[29].

Table 1 Screening of cardiovascular risk in asymptomatic individuals (based on References[6,7])

Class of recommendation	Screening tools	Level of evidence
Class I	Risk prediction by scores and family history	B
Class IIa	hs-CRP in men > 50 yr or women > 60 yr of age without other risk factors, to select for statin therapy	B
	ECG at rest if there are risk factors such as diabetes/hypertension	C
	Carotid intima-media thickness if intermediate-risk	B
	Coronary calcium if intermediate-risk[1]	B
Class IIb	HbA1c if asymptomatic adults without a diagnosis of diabetes	
	hs-CRP if asymptomatic intermediate-risk	B
	ECG at rest without risk factors	C
	Exercise ECG if intermediate risk, including sedentary adults considering vigorous exercise	B
	Resting echocardiography if hypertension (to look for left ventricular hypertrophy)	B
	Myocardial perfusion imaging for asymptomatic adults with diabetes, strong family history, or high risk in previous investigations	C
	Coronary calcium if low-intermediate risk, not recommended for low risk	B
Class III	Genetic prediction	B
	Lipoproteins, apolipoproteins, particle size/density beyond a standard fasting lipid profile	B
	Peripheral arterial flow-mediated dilation	B
	Stress echocardiography if low/intermediate-risk (primarily used for symptomatic patients, to know prognosis in a known coronary artery disease)	C
	Myocardial perfusion imaging if low/intermediate-risk diabetes, strong family history, or high risk in previous investigations	C
	CT coronary angiography	C
	MRI of plaque	C

[1]Generally not present in men < 40 yr or women < 50 yr of age, the ideal score is 0, no ideal cutoff, risk increases with the increasing score, adds to the prediction of Framingham risk score.
CT: Computed tomography; ECG: Electrocardiography; hs-CRP: High-sensitivity C-reactive protein; MRI: Magnetic resonance imaging.

Exercise: Achieving physical activity at any level or increasing level over the past is better than continuing sedentary behavior. Recommended physical activity consists of at least 2.5 h of moderate-intensity exercise or 75 min of vigorous-intensity exercise per week, supplemented by less sedentary time and at least two sessions of muscle-building exercise. For improving overall cardiometabolic health, particularly resistance training has beneficial effects by changing body composition which promotes anti-atherogenic and anti-inflammatory mechanisms[30].

Dietary changes: A Mediterranean diet rich in vegetables, fruits, legumes, nuts, beans, cereals, grains, fish, and unsaturated fats such as olive oil with and low-carbohydrate diet < 30 g/d are recommended and this special diet strategy has been shown to significantly improve both liver fat content and improve cardiometabolic risk parameters[31]. Additionally, 'when' and 'how often' to eat is also important as well as what we eat[6]. Receiving most of the calories before bedtime are associated with a higher risk of NAFLD. At meantime, shifting most of the daily calorie intake for the breakfast or implementing intermittent fasting with energy restriction for about 10-16 h seems to be beneficial for obtaining the ideal body weight[32]. Additional lifestyle modifying interventions includes learning strategies to simulate the physiologic response to distress and restorative sleep patterns which have downstream effects on the endocrine pathways relevant in the development of cardiometabolic diseases[33].

Pharmacological interventions

Glucose-lowering agents in NAFLD patients with concomitant type 2 diabetes mellitus: Some glucose-lowering medications promote the accumulation of ectopic fat

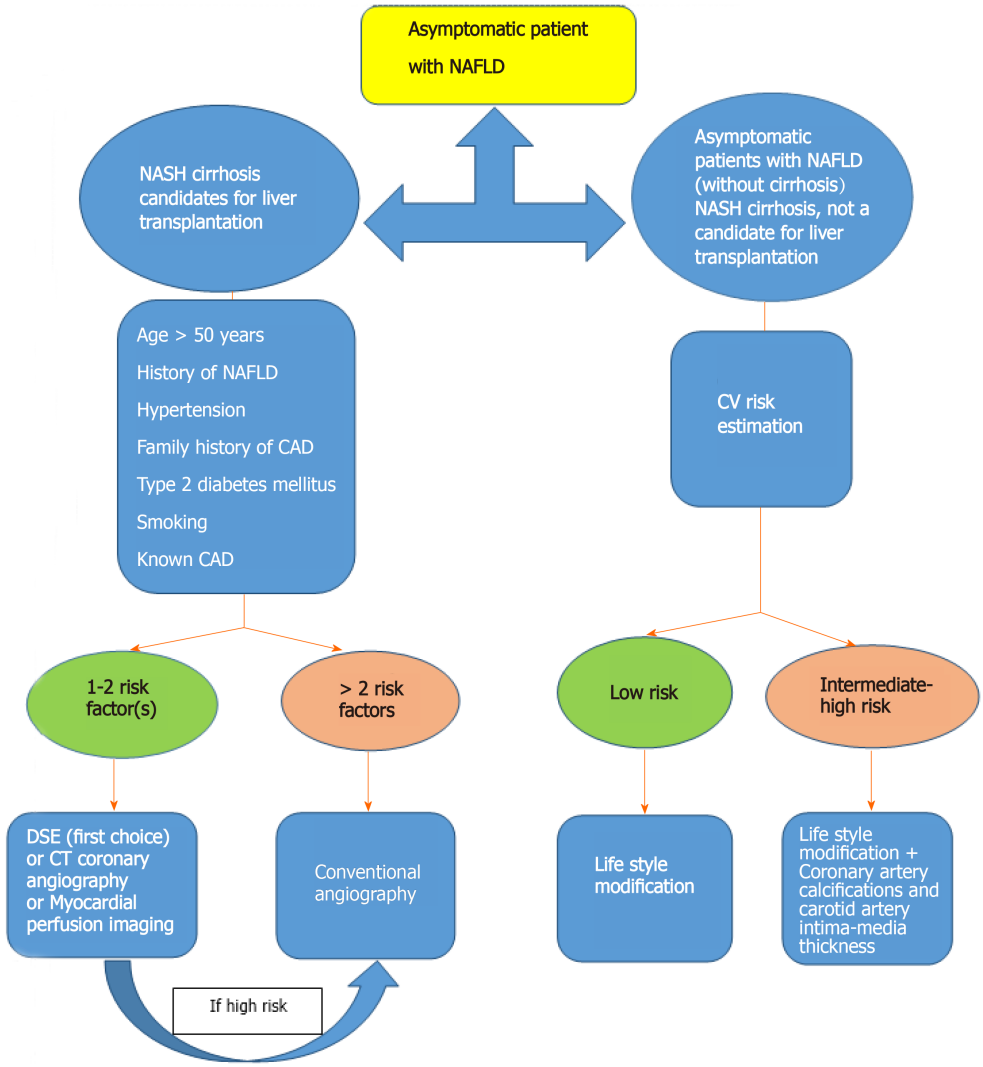


Figure 3 Proposed algorithm for the screening of cardiovascular disease in patients with nonalcoholic fatty liver disease. CVD: Cardiovascular disease; CT: Computed tomography; DSE: Dobutamine stress echo; NAFLD: Nonalcoholic fatty liver disease.

(*e.g.*, insulin/insulin analogs, sulfonylureas), while other antidiabetic medications [*e.g.*, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 (GLP-1) receptor] decrease the accumulation of ectopic fat without reducing inflammation. In contrast, both SGLT2 (sodium-glucose-linked transporter 2) inhibitors and metformin reduce inflammation, the quantity of ectopic fat and secretion of adipokines. Especially, the novel anti-diabetic drugs such as SGLT2 inhibitors and GLP-1 analogs represent good choices, by providing a favorable influence on the pathogenesis of renal, hepatic and cardiac disease, even in non-diabetic patients[6,34].

Statins: Statins have anti-inflammatory, anti-oxidative, anti-fibrotic, and plaque-stabilizing properties, which make them a promising choice to improve vascular and hepatic function among NAFLD patients and reduce cardiovascular risk. The major guidelines on statins have not addressed the specific NAFLD population yet, but it may be reasonable to consider a similar approach as to those with diabetes[5]. According to the recommendations of practice guideline provided by the American College of Gastroenterology, American Association for the Study of Liver Diseases, and the American Gastroenterological Association for the diagnosis and management of NAFLD, statins can be used to treat dyslipidemia in patients with NASH and NAFLD[35]. The Liver Expert Panel suggests that statin use can be safe in NASH and NAFLD and there is no need for routine liver enzyme measurements. However, checking liver enzymes is recommended before starting statin therapy[36].

Agents directed at the renin-angiotensin-aldosterone system: Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers have been noted as a promising drug in NAFLD patients with hypertension since the renin-angiotensin-

aldosterone system is involved in the pathogenesis of both NAFLD and CVDs. Although protective effects of renin-angiotensin system blockers on histological fibrosis progression in NAFLD have been demonstrated in several reports, the use of renin-angiotensin system blockers is not recommended in NAFLD patients without hypertension based on the current evidence[37].

Acetylsalicylic acid: In the context of CVD and NAFLD concatenation, acetylsalicylic acid (ASA) is a promising approach. Regarding the benefits of ASA use for primary CVD prevention, there is contradicting evidence; in a recent meta-analysis and systematic review evaluating 13 trials and randomizing (164,225) people to daily aspirin as primary prevention of CVD showed it to contribute to a lower risk of adverse cardiovascular events but a higher risk of major bleeding[38]. ASA is recommended as secondary prevention in all patients with established ASCVD including coronary, cerebral, or peripheral arterial disease. Moreover, a recent prospective cohort study suggests that ASA may also have antifibrotic effects in NAFLD, especially in patients who received daily ASA for ≥ 4 years[39]. Although, the underlying hepatoprotective benefit is unclear. Meanwhile, reports exists about the beneficial effect of ASA use in inhibition of liver cell fibrosis[40].

CONCLUSION

Patients with NAFLD have a high risk for adverse cardiovascular events and mortality. As such, the diagnosis of NAFLD deserves a thoughtful cardiovascular risk assessment and evaluation for subclinical atherosclerosis to prevent CVD morbidity/mortality. In patients with NASH-related cirrhosis undergoing liver transplantation, screening for significant cardiovascular arterial disease should be done by stress echocardiography and CT coronary angiography, as first-line and conventional angiography is reserved for those with the combination of risk factors for cardiovascular arterial disease.

Recommendation for screening in asymptomatic NAFLD without significant fibrosis is not clear, with each modality having some limitations. When NAFLD is diagnosed, the CVD risk assessment must be sought out and patients with intermediate or high risk or with NASH cirrhosis should be referred to the cardiologist for evaluation of CVD.

The basis of NAFLD prevention and treatment is lifestyle modifications, including weight loss, increased physical activity, and a Mediterranean/low-carbohydrate diet. While no pharmacologic therapy for the liver itself is approved yet, concomitant cardiovascular risk factors should be targeted by using statins, ACE inhibitors and angiotensin-receptor blockers, aspirin, and insulin-sensitizing agents like GLP-1 analogs or metformin and probably SGLT2 inhibitors.

ACKNOWLEDGEMENTS

The authors would like to show their appreciation to the Staff of Sina Hospital Research Development Center for their technical support in preparing the draft.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 April 28; 9(2): 176-192

DOI: 10.13105/wjma.v9.i2.176

ISSN 2308-3840 (online)

MINIREVIEWS

Therapeutic applications of dental pulp stem cells in regenerating dental, periodontal and oral-related structures

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Author contributions: Grawish ME conceptualized the idea, wrote and reviewed the manuscript; Saeed MA, Sultan N and Scheven BA contributed to writing of the manuscript and provided intellectual contribution to the content; All authors reviewed and approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests for this article.

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Abstract

Dental pulp stem cells (DPSCs) have emerged as a promising tool with great potential for use in tissue regeneration and engineering. Some of the main advantages of these cells are their multifaceted differentiation capacity, along with their high proliferation rate, a relative simplicity of extraction and culture that enables obtaining patient-specific cell lines for their use in autologous cell therapy. PubMed, Scopus and Google Scholar databases were searched for relevant articles related to the use of DPSCs in regeneration of dentin-pulp complex (DPC), periodontal tissues, salivary gland and craniomaxillofacial bone defects. Few studies were found regarding the use of DPSCs for regeneration of DPC. Scaffold-based combined with DPSCs isolated from healthy pulps was the strategy used for DPC regeneration. Studies involved subcutaneous implantation of scaffolds loaded with DPSCs pretreated with odontogenic media, or performed on human tooth root model as a root slice. Most of the studies were related to periodontal tissue regeneration which mainly utilized DPSCs/secretome. For periodontal tissues, DPSCs or their secretome were isolated from healthy or inflamed pulps and they were used either for preclinical or clinical studies. Regarding salivary gland regeneration, the submandibular gland was the only model used for the preclinical studies and DPSCs or their secretome were isolated only from healthy pulps and they were used in preclinical studies. Likewise, DPSCs have been studied for craniomaxillofacial bone defects in the form of mandibular, calvarial and craniofacial bone defects where DPSCs were isolated only from healthy pulps for preclinical and clinical studies. From the previous results, we can conclude that DPSCs is promising candidate for dental and oral tissue regeneration.

Manuscript source: Invited manuscript

Specialty type: Dentistry, oral surgery and medicine

Country/Territory of origin: Egypt

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E

Received: February 2, 2021
Peer-review started: February 2, 2021
First decision: March 8, 2021
Revised: March 13, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Kannan T, Tsutsui TW
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li JH



Key Words: Dental pulp stem cells; Dentin-pulp complex; Periodontal tissues; Salivary glands; Cell-based therapy; Cell-free therapy

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Core Tip: Dental pulp stem cells (DPSCs) can be isolated from the pulps of impacted teeth requiring surgical extraction or from teeth extracted for orthodontic reasons. In addition, there is growing evidence that DPSCs can be isolated from inflamed pulps derived from carious teeth with symptomatic irreversible pulpitis. DPSCs are neural crest in origin, and thus they differentiate into an astonishing array of cell types and tissues and regenerate structures that have the same developmental origin, like dentin-pulp complex, periodontal tissues, stromal tissues of salivary glands and craniomaxillo-facial bone defects.

Citation: Grawish ME, Saeed MA, Sultan N, Scheven BA. Therapeutic applications of dental pulp stem cells in regenerating dental, periodontal and oral-related structures. *World J Meta-Anal* 2021; 9(2): 176-192
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/176.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.176

INTRODUCTION

The oral cavity is a complex multi-organic structure, where oral tissues and organs are linked developmentally and functionally. Irreversible damage to any of them is more likely to affect the others, causing extensive malfunction. Tooth decay, periodontal diseases, alveolar bone resorption, and impaired salivary gland (SG) function are conditions that seriously affect oral health. Synthetic materials have traditionally been used as the treatment of choice in prosthetic and restorative dentistry. However, this form of replacement does not regenerate the original biological tissues and other organs, such as SGs, are simply not responsive to mechanical substitution approaches. Thus, tissue engineering (TE) is emerging as a new therapeutic choice for the complete biological regeneration of craniomaxillofacial tissues and organs. Three critical components are required: (1) stem cells; (2) scaffolds; and (3) stimulating factors or inductive signals[1].

Dental pulp stem cells (DPSCs) are neural crest-derived cells with an excellent ability to differentiate along multiple cell lineages (Figure 1). In particular, simple *in vitro* protocols can be used to achieve highly efficient osteo/dentinogenic differentiation of DPSCs, making these cells a very attractive and promising tool for the future treatment of dental and periodontal diseases. Among oral-related structures, regeneration of the SGs and bone defects appears to be possible using DPSCs, as researchers over the last decade have made substantial progress in experimental models of partial or total regeneration of both organs[2]. Emerging approaches using cell-free therapy with cell extracts or secretome components also exhibit favorable outcomes and when compared to cell-based approaches, the secretome can be relatively easily obtained, quantified and are more stable for long-term storage to be used in various TE fields[3].

In this review, we explored the potential usage of DPSCs in preclinical and clinical trials for the regeneration of different oral, dental and craniomaxillofacial tissues and organs. Therefore, PubMed, Scopus and Google Scholar databases were searched for relevant articles related to the use of DPSCs in regeneration of dentin-pulp complex (DPC), periodontal tissues, SG and craniomaxillofacial bone defects. We present the different strategies used in experimental regenerative models with potential applications in dentistry and oral medicine. Based on data extraction from the different databases, we can conclude that DPSCs may be an ideal choice to be used in experimental models of tissue-engineered reconstruction of organs of the oral cavity, and the paradigmatic cases of the tooth and the SG may constitute the leading edge of a massive research effort on next generation organ replacement therapies.

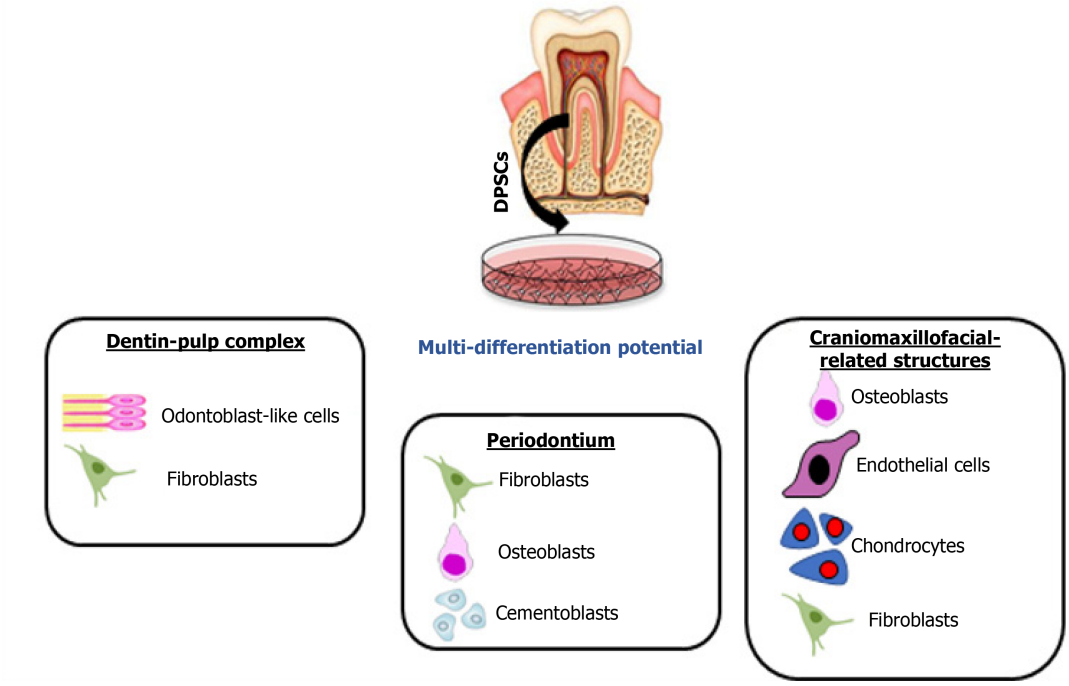


Figure 1 Different types of regeneration possible from dental pulp stem cells. Schematic diagram showing multi-differentiation capability of dental pulp stem cells (DPSCs) for regenerating dentin-pulp complex, periodontal tissues, stromal tissues of salivary glands and craniomaxillofacial bone defects.

REGENERATION OF DPC USING DPSCS

Teeth are composed of various dental tissues, *viz*. enamel, dentin, pulp, and cementum. Dentin and pulp formulate the DPC that preserves tooth viability and integrity[4]. On one hand, the pulp provides dentin with moisture, nutriment, and minerals to keep its resiliency and degree of mineralization; therefore, in the case of pulp necrosis, dentin gets dark and brittle with the sequelae of tooth discoloration and fracture. On the other hand, dentin provides a closed protecting barrier for the pulp against bacterial toxins and physical or chemical irritants; therefore, dentin damage or pulp exposure seriously threaten tooth vitality and integrity[5].

Following carious or traumatic injury to dentine-pulp, a complex interplay between host defense response, inflammatory and infective agents and endogenous repair processes occur. DPC is a difficult structure to manufacture *in vitro* or repair *in vivo* or *ex vivo*. Embryologically, dentinogenesis starts with the secretion and cross basement membrane transport of signaling proteins from the differentiating ameloblasts during the early bell stage of tooth development. Amongst many cytokines and growth factors, the TGFβ is considered to initiate the dentinogenesis process through inducing odontogenic differentiation of the undifferentiated ectomesenchymal cells of the dental papilla[6]. During development and after enamel maturation and tooth eruption, ameloblasts are lost forever; therefore, recreating the dentinogenesis process again in TE without ameloblasts is difficult due to the absence of the initiator. Histologically, the DPC is formed of two different tissues. The outermost layer of the dental pulp (DP) consists of odontoblasts that responsible for the formation of primary, secondary and tertiary dentin throughout life. The odontoblastic processes may extend from the odontoblasts through the whole thickness of dentin and may be embedded in the enamel as enamel spindles, dentin includes various noncollagenous proteins that control the activity of odontoblasts, fibroblasts and DPSCs[7].

Several biomaterials are used to repair the dentin and regenerate DPC, such as calcium hydroxide, adhesive resin cement, mineral trioxide aggregate, Biodentine, and emdogain. Actually, all these materials are developed on empirical bases, rather than on the deep understanding of the histological structure of the DPC or the molecular bases of the pulp self-healing mechanism[8]. Consequently, the results of these previous studies showed the inability of these materials to regenerate the DPC, and only disorganized tissues were formed as a response[9].

The idea of regenerating dentin and pulp tissues is based on recruiting DPSCs *via* several growth factors and scaffolds, what is known as the biological triad of tissue regeneration. The DPSCs have a crucial role in the dentinogenesis process as they are

responsible for the reconstruction of the damaged dentin and pulp. These cells work under the influence of chemical/physical signaling mechanisms that induce stem cell differentiation to express the desired phenotype[10]. DPSCs were successfully tested in preclinical and clinical studies for DPC regeneration.

Preclinical studies

DPSCs[11-14] and DP progenitor cells[12] have been isolated from permanent teeth pulps. In some instances, the cells were transfected with lentivirus virus-mediated gene transfer of platelet-derived growth factor (PDGF)[15]. The strategy of using DPSCs was dependent on scaffold-based and scaffold-free therapies. DPSCs were loaded into the scaffold[12,13] or, incorporated into the scaffold and the combination was injected locally[11,14,15]. Nude mice and minipigs were the most selected model for studying the regenerative effect of DPSCs for DPC regeneration in the pre-clinical trials. The cell number most commonly implicated was 1×10^6[11-13] and an 8×10^6[14] cell number was also used (Table 1).

DPSCs-derived from healthy pulp: Loading or injecting a cell suspension of DPSCs. Horibe *et al*[13] investigated the regenerative capacity of young and aged human (h)DPSCs on ectopic pulp regeneration. They injected 1×10^6 cells combined with pepsin-solubilized collagen solution derived from porcine skin into the human tooth root model prior to subcutaneous transplantation into 5-wk-old severe combined immunodeficiency mice. Evaluation was dependent on histologic and histomorphometric analysis, 28 d after transplantation. They concluded that both young and old hDPSCs are promising for clinical applications in DP regeneration.

Wang *et al*[11] seeded 1×10^6 hDPSCs on nanofibrous poly(L-lactic acid) (PLLA) scaffolds, 5.2 mm in diameter and 1.5 mm in thickness. The cell-scaffold constructs were induced in odontogenic media for 2 wk prior to subcutaneous implantation in nude mice with an age 6-8 wk. A cell-scaffold construct cultured in media without odontogenic inducers was used as the control group. The endpoint for this experiment was 8 wk after implantation. The histological examinations of the specimens illustrated formation of new blood vessels and collagen. The immunohistological examin-ation showed high expression of dentin sialoprotein that indicating odontogenic differentiation of hDPSCs.

Pan *et al*[12] investigated the potential of the DP progenitors to regenerate DPC and, besides, they clarified the efficacy of stem cell factor (SCF) on DP progenitors for odontogenic differentiation and regenerative capability. They mixed 1×10^6 DP progenitors with 3 mm × 3 mm × 3 mm collagen sponge sheets that were coated with 50 ng/mL SCF aqueous solution for a preclinical study. The other groups used in this study were collagen sponges with DP progenitors, collagen sponges with SCF, and collagen sponges only as a control. The cell-scaffold constructs were implanted into the back of male nude mice for 4 wk. After the endpoint of the experiment, the collagen sponges were dissected and prepared for histological examination. The results of this study revealed a high potentiality of the DP progenitors in DPC regeneration. In addition, using the SCF increased the regenerative capacity of DP progenitors.

Chaudhary *et al*[14] synthesized nanofibrous sponge microspheres (NF-SMS), which were used to carry hDPSCs into the pulp cavity to regenerate living DP tissues. They removed the DP from 16 molars collected from 2-wk-old rabbits. After that, they injected 8×10^6 hDPSCs/NF-SMS complexes pre-cultured under hypoxic condition into the rabbit molar pulp cavities prior to subcutaneous implantation into nude mice with age of 6-8 wk, for 4 wk. The results of this study were evaluated histologically and immunohistochemically. The authors concluded that the hypoxia group significantly enhanced angiogenesis inside the pulp chamber and promoted the formation of odontoblast-like cells lining the dentin-pulp interface, as compared to the control groups, hDPSCs group, NF-SMS group, and hDPSCs/NF-SMS group pre-cultured under normoxic conditions.

Zhang *et al*[15] explored the influence of the PDGF-BB on hDPSCs proliferation and odontogenic differentiation. They established a PDGF-BB gene-modified hDPSCs using lentivirus delivery vector. The genetically-modified hDPSCs were mixed with a porous calcium phosphate cement scaffold and implanted subcutaneously in nude mice for 12 wk. DPC regeneration was evaluated using histological and immunohistochemical analysis. The conclusion of this study was that the PDGF-BB gene-modified hDPSCs can continuously secrete the PDGF-BB protein enhancing dentin-like tissue formation. The formed dentin-like mineralized tissue showed positive staining for the dentin sialophosphoprotein, similar to tooth dentin tissue, and was surrounded by highly vascularized DP-like connective tissue.

Table 1 Preclinical studies obtained from the PubMed, Scopus and Google Scholar databases related to dentin-pulp complex regeneration using dental pulp stem cells

Ref.	Strategy	Model	Defect/site of cell implantation	Cell number	Cell source	Cell application
Wang *et al*[11], 2011	Scaffold-based	Nude mice	Subcutaneous implantation	1×10^6	hDPSCs	Loaded on the top surface of the scaffold
Pan *et al*[12], 2013	Scaffold-based	Nude mice	Subcutaneous implantation	1×10^6	DP progenitors	Mixed with collagen sponge sheets and SCF
Horibe *et al*[13], 2014	Scaffold-based	SCID mice	Subcutaneous implantation	1×10^6	hDPSCs	Loaded into human tooth root model
Chaudhary *et al*[14], 2016	Scaffold-based	Nude mice/ nude rats	Subcutaneous implantation/ first molar root canal	8×10^6	hDPSCs	Loaded into rabbit molar pulp cavity/ cell injection into root canals
Zhang *et al*[15], 2017	Scaffold-base	Nude mice	Subcutaneous implantation	Not detected	hDPSCs	Mixed with porous calcium phosphate cement

hDPSCs: Human dental pulp stem cells; SCF: Stem cell factor; SCID: Severe combined immunodeficiency.

In another experiment, Chaudhary *et al*[14] used the maxillary first molars of the nude rats as an *in situ* pulp regeneration model. The entire pulp was removed and the root canals were cleaned and shaped using manual #15-#25 K files. After irrigation with phosphate-buffered saline and 2.25% NaClO and drying the root canals with absorbent points, 8×10^6 hDPSCs/NF-SMS complexes primed under hypoxic conditions were injected into the prepared root canals, while those primed under normoxic conditions were injected into prepared contralateral first molar root canals. After 4 wk, the treated maxillary molars were harvested. For comparison, maxillary first molars received no treatment after pulp removal. The histological and immunohistochemical analyses of the specimens revealed that the hypoxia-primed hDPSCs/NF-SMS could fully fill the molar canals through injection and promoted pulp-like tissue formation with intimate integration with the native dentin.

REGENERATION OF PERIODONTAL TISSUES USING DPSCS

The periodontal tissues include two hard and two soft tissues. The two soft tissues are periodontal ligament and gingiva, while the two hard ones are alveolar bone lining the socket and cementum. The principal function of these structures is investing and supporting the teeth to perform their physiologic function. The most common cause for tooth loss in adults is the periodontitis, affecting 10%-15% of adults worldwide as an advanced type. Treatment modalities such as bone grafts[16], enamel matrix derivative[17], platelet-rich plasma (PRP)[18], and guided tissue regeneration[19] allow partial improvements in the architecture and function of the periodontal tissues but fail to completely regenerate this complex structure. Procedures attempting to regenerate the periodontal apparatus have become unconventional and more advanced to include dental tissue-derived stem cell therapy[20]. Chiefly, there are two approaches for periodontal tissues regeneration, cell-based and cell-free therapies. The major concern for the cell-based therapy is the complicated implantation process and it includes stem cell injection of cell suspension and stem cell sheet transplantation techniques. Engineering of a cell sheet has been adopted through utilizing cell culture dishes of temperature-responsive surfaces[21], or by culturing the cells in ascorbic acid[22]. Even though cultured cells can be used to study the pathogenesis of the inflammatory and immune pathways of periodontal diseases, the complicated host defense system basically responsible for these diseases is not amenable to being reproduced *in vitro*. Experimental periodontitis is a preclinical *in vivo* model for studying the possible application of cell therapies and biomaterials-targeted for periodontal tissues regeneration. They were considered to be more appropriate mimicking the natural host immune response under microbial loads, which can cause oral pathological environments and periodontal diseases[23]. Animals like nonhuman primates, rodents, rabbits, pigs, and dogs have been selected for modeling human periodontitis. Commonly, periodontitis has been induced by placing a silk ligature inside gingival sulcus around the molar teeth to serve as a retentive factor for plaque and microbial proliferation. In addition, alveolar bone loss has been induced by

injection or inoculation of human oral bacteria[24]. DPSCs were successfully tested in preclinical and clinical studies for periodontal tissues regeneration.

Preclinical studies

Xenogeneic and autologous[25-32] DPSCs or their exosomes (Exo)[32] have been isolated from healthy[25-28,30-32] or inflamed pulps[29]. In some instances, the cells were transfected with adenovirus-mediated gene transfer of hepatocyte growth factor (HGF)[27]. The strategy of using DPSCs was dependent on scaffold-based[25,29,30,32], sandwich structure[31], scaffold-free[26-28] and cell-free[32] therapies. Application of DPSCs was in the form of cell injection[26-28,30], cell sheet transplantation[27,28,31], loaded onto the scaffold[25,29] or, incorporated onto the scaffold and the combination was injected locally[32]. Miniature pigs were the most selected model for studying the regenerative effect of DPSCs for periodontal tissues regeneration in the pre-clinical trials[26-29]. Other animals such as rats[30], mice[31,32] and dogs[25] were also selected for the same purpose. Bone defects of 5 mm width, 7 mm length and 3 mm depth[26,27] and of 5 mm × 6 mm × 5 mm[28] were the most common surgical periodontal defect models created as a site of cell implantation (Figure 2). Ligature-induced periodontitis with three-walled periodontal defects (PDs)[25], PDs adjacent to the root of the second molars[29], periodontitis created using 5-0 silk ligature[32] were also applied as models. Bioroot regeneration was accomplished through subcutaneous transplantation of a sandwich structure constituted by hDPSCs sheet, human treated dentin matrix (hTDM)/hDPSCs and Matrigel/hDPSCs[31]. The cell number of 1 × 10^7 was the most commonly implicated[26-29], and 5 × 10^5[31], 2 × 10^6[30], 2 × 10^7[25] cell numbers were also used; in addition, 50 μg[32] of hDPSCs-Exo was indicated for the cell-free therapy strategy (Table 2).

DPSCs-derived from healthy pulp: (1) Loading or injecting a cell suspension of DPSCs. In a scaffold-based trial, Khorsand *et al*[25] surgically created three-walled PDs with ligature-induced periodontitis in 10 mongrel dogs. The PDs were produced bilaterally at the bone adjacent to the mesial surface of the mandibular first premolar teeth. They investigated the regenerative capacity of autologous dogs' DPSCs on periodontal tissues, with/without Bio-Oss granules. Four weeks after making the periodontitis model, Bio-Oss was engrafted on one side and considered as a control, while on the other side, a combination of 2 × 10^7 autologous DPSCs of passage 3 with Bio-Oss was engrafted and served as the test group. Evaluation was dependent on histologic and histomorphometric analyses, 8 wk after surgery, in terms of cementum, periodontal ligament, and bone formation. The authors concluded that a biocomplex consisting of Bio-Oss scaffold and DPSCs would be advantageous in regeneration of periodontal tissues. Moreover, Lin *et al*[30] investigated the regenerative and therapeutic effects of a daily dose, intragastrically administered 25 mg/kg of 2,3,5,4'-tetrahydroxystilbene-2-O-β-d-glucoside (THSG)-triggered hDPSCs in the healing of experimental PDs adjacent to the roots of the second molars in male Sprague-Dawley rats. THSG is a water soluble and biologically active component of the Chinese herbal medicine *Polygonum multiflorum*. The defects of the negative control were treated with Matrigel, while the defects of the positive control received 2 × 10^6 hDPSCs/ Matrigel (vol/vol = 3:1). It was found that THSG revolutionized hDPSCs significantly and shortened the regenerative period of PDs by enhancing new bone formation and increasing expression levels of osteopontin, vascular endothelial growth factor, and proliferating cell nuclear antigen than those in other groups.

In a scaffold-free trial, Ma *et al*[26] evaluated the injection of DPSCs or bone marrow stem cells (BMSCs) for *in vivo* periodontal regeneration. The investigators created 12 PDs of 3 mm depth, 7 mm length, and 5 mm width in miniature pigs' interradicular bone of the first molars. The defects were divided into three groups, namely DPSCs, BMSCs and control group, each with four defects. The BMSCs and DPSCs groups were separately injected with approximately 1 × 10^7 human cells in 0.6 mL of 0.9% NaCl, while the defects of the control group were injected with 0.9% NaCl as the cell injection group. Lymphocyte infiltration and impaired sulcular epithelia were evident in the control group but not in the DPSCs and BMSCs groups. Notably, the periodontal bone regeneration capacity was significantly greater for the DPSCs injection group compared to the BMSCs group.

(2) Cell sheet transplantation technique of DPSCs. In a scaffold-based trial, Meng *et al*[31] created a sandwich structure of hDPSCs sheet/hTDM/Matrigel to be subcutaneously transplanted in nude mice for 3 mo. The main objective was to investigate the regeneration potential of hDPSCs, and the characteristics of the above materials for the tooth root regeneration (bioroot). The hDPSCs sheet was obtained by

Table 2 Preclinical and clinical studies obtained from the PubMed, Scopus and Google Scholar databases related to periodontal tissue regeneration using dental pulp stem cells

Ref.	Strategy	Model	Defect/site of cell implantation	Cell number	Cell source	Cell application
Khorsand *et al*[25], 2013	Scaffold-based	Mongrel dogs	3-walled PDs with ligature induced periodontitis	2×10^7	Autologous dogs' DPSCs	Loaded on the top surface of the scaffold
Ma *et al*[26], 2014	Scaffold-free	Miniature pigs	Bone defect of 5 mm width, 7 mm length, and 3 mm depth	1×10^7	hDPSCs	Cell injection
Cao *et al*[27], 2015	Scaffold-free	Miniature pigs	Bone defects of 5 mm width, 7 mm length, and 3 mm depth	1×10^7	hDPSCs transfected with adenovirus-mediated transfer of HGF gene	Cell sheet transplantation or cell injection
Hu *et al*[28], 2016	Scaffold-free	Miniature pigs	Bone defect of 5 mm width, 7 mm length, and 3 mm depth	1×10^7	hDPSCs	Cell sheet transplantation or cell injection
Li *et al*[29], 2019	Scaffold-based	Miniature pig	Periodontal defect of 5 mm × 6 mm × 5 mm	1×10^7	Autologous pig DPSCs-IPs	loaded onto the scaffold
Lin *et al*[30], 2020	Scaffold-based	Sprague-Dawley rats	Periodontal defect adjacent to the root of the second molars	2×10^6	hDPSCs	Cell injection
Meng *et al*[31], 2020	Sandwich structure	Nude mice	Subcutaneously (bioroot regeneration)	5×10^5	hDPSCs	Cell sheet
Shen *et al*[32], 2020	Scaffold-based/cell-free therapy	Mouse	Periodontitis created using 5-0 silk ligature	50 μg	hDPSCs-Exo	Incorporated onto the scaffold and combination injected locally
Li *et al*[33], 2016	Scaffold-based	Human	Periodontal intrabony defects	1×10^5	Autologous DPSCs-IPs	Loaded onto the scaffold material
Ferrarotti *et al*[34], 2018	Scaffold-based	Human	Chronic periodontitis with deep intrabony defect	50 μm micrograft	Micrografts rich in autologous hDPSCs	Seeded onto the scaffold
Aimetti *et al*[35], 2018	Scaffold-based	Human	Chronic periodontitis with deep intrabony defect	50 μm micrograft	Micrografts rich in autologous hDPSCs	Loaded on the scaffold
Hernández-Monjaraz *et al*[36], 2020	Scaffold-based	Human	PDs, without uncontrolled systemic chronic diseases	5×10^6	hDPSCs	Dripped suspended in 200 mL of PBS and then collagen membrane was placed

DPSCs: Dental pulp stem cells; hDPSCs: Human dental pulp stem cells; HGF: Hepatocyte growth factor; IPs: Inflammatory dental pulp tissues; PBS: Phosphate-buffered saline; PDs: Periodontal defects.

seeding 5×10^5 hDPSCs in a culture dish containing complete medium supplemented with 20.0 μg/mL vitamin C. The hTDM was fabricated from healthy extracted teeth and a disk with 5 mm thickness was prepared to be successively decalcified and sterilized. The hTDM/hDPSCs complex was prepared by seeding hDPSCs on hTDM and cultured *in vitro* for 2 d, and the Matrigel/hDPSCs suspension was injected into the complex's pulp cavity. It was concluded that hDPSCs could be used as seed cells for the whole tooth root regeneration, and the sandwich structure constituted by hDPSCs sheet, hTDM/hDPSCs and Matrigel/hDPSCs could be utilized for tooth root regeneration.

In a scaffold-free trial, Cao *et al*[27] created 40 PDs of 3 mm depth, 7 mm length, and 5 mm width in miniature pigs, in the mesial region of the mandibular and maxillary first molars. Under good manufacturing practice, hDPSCs were transfected with adenovirus-mediated gene transfer of HGF to evaluate their potential roles for periodontal tissues regeneration. They found that injecting HGF-hDPSCs could significantly improve soft tissue healing and periodontal bone regeneration. In comparison to the injection of dissociated cells, HGF-hDPSCs and hDPSCs sheets showed superior periodontal tissue regeneration. The authors attributed their findings to an increase in blood vessel regeneration and a marked decrease of hDPSCs apoptosis caused by adenovirus-mediated gene transfer of HGF. They concluded that sheets are more suitable for the surgically-managed periodontitis treatments, as they required surgical placement. Moreover, Hu *et al*[28] created 24 PDs of 3 mm depth, 7 mm length and 5 mm width in the mesial region of the mandibular and maxillary first molars of miniature pigs. The hDPSCs were applied using techniques of cell sheet

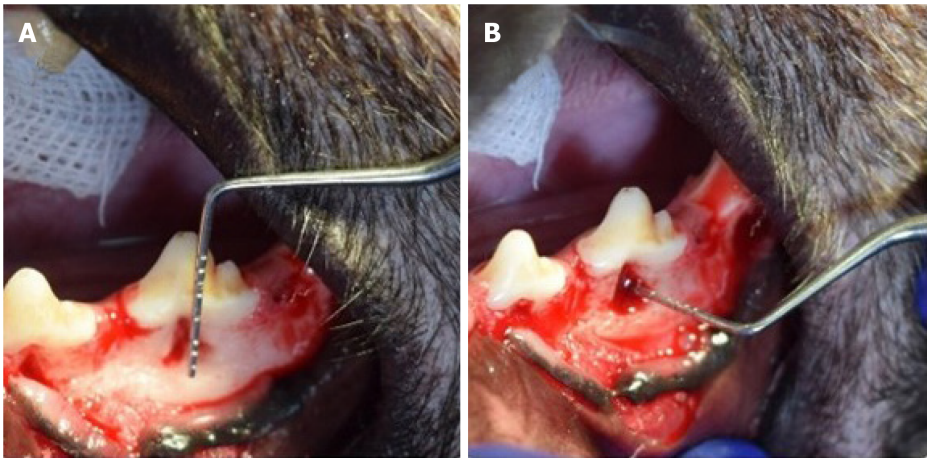


Figure 2 Periodontal bone defect. A and B: Photographs showing the periodontal defect of vertical height and horizontal width and depth of a dog's interradicular bone of the first molar. Courtesy provided by the staff members of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.

transplantation or cell injection. The complete form of a morphologically cell sheet was produced by inducing hDPSCs with 20.0 μg/mL vitamin C. The cells of the cell sheet contacted with each other tightly and contained five or six layers. Both cell sheet transplantation and hDPSCs injection significantly regenerated soft tissues and periodontal bone. Compared with hDPSCs injection, the hDPSCs sheet had more bone regenerative capacity.

Finally, (3) secretome-derived from DPSCs. In a scaffold-based trial, Shen *et al*[32] created a periodontitis mouse model using 5-0 silk ligature tied around the maxillary left second molar to evaluate the effect of chitosan (CS) hydrogel incorporated with hDPSCs-derived Exo. For Exo treatment, an equal volume of phosphate-buffered saline /CS, hDPSCs-Exo (50 μg), and hDPSCs-Exo/CS was injected locally after ligature removal. It was found that treatment with hDPSCs-Exo/CS alleviated periodontitis *via* macrophage-dependent mechanism through miR-1246. hDPSCs-Exo facilitated the way for the macrophages to be converted from a pro-inflammatory phenotype to an anti-inflammatory phenotype, and suppressed periodontal inflammation and modulated the immune response.

DPSCs-derived from inflamed pulp: In a scaffold-based trial, Li *et al*[29] cultured an autologous miniature pig DPSCs derived from inflammatory dental pulp tissues (IPs). The cells were characterized and loaded onto β-tricalcium phosphate (β-TCP) biomaterial. The compounds were then engrafted into artificially-created PDs of 5 mm × 6 mm × 5 mm in the buccal region of root furcations of the third premolars and first molars. Compared to the β-TCP and control groups, DPSCs-IPs expressed moderate to high levels of CD146 and STRO-1 as well as low levels of CD45 and CD34. The cells have chondrogenic, adipogenic, and osteogenic differentiation abilities and when they were engrafted onto β-TCP, they regenerated bone of the PDs through 3 mo post-surgical reconstruction.

Clinical studies

Autologous hDPSCs-IPs[33], micrografts rich in autologous hDPSCs[34,35] or autologous hDPSCs derived from healthy pulps[36] were the source for a randomized clinical trial (commonly referred to as RCT)[34], a 1-year follow-up case series[35], a quasi-experimental study[35] and a pilot study[33]. The strategy of using DPSCs was dependent on scaffold-based only[33-35] as they loaded onto the scaffold[33-35] or dripped suspended in 200 mL of phosphate-buffered saline and then the scaffold membrane was placed[36]. Patients diagnosed with combined periodontal-endodontic lesions with pocket depth from 5 mm to 6 mm[33], patients with chronic periodontitis combined with deep intrabony defects[34,35], and patients with controlled chronic diseases and without horizontal PDs or osteoporosis[36] were the models used for cell implantation. Either 1×10^5[33] or 5×10^5[36] and 50 μm micrografts rich in autologous hDPSCs were the cell number/source that was indicated for the scaffold-based strategy (Table 2).

DPSCs-derived from healthy pulp: In a scaffold-based trial, Ferrarotti *et al*[34] performed a RCT for 29 consecutively enrolled patients with chronic periodontitis

presenting one deep intrabony defect and requiring extraction of one vital tooth. They randomly allocated the defects to two groups, test or control ones. They prepared micrografts rich in autologous DPSCs from the DP of the extracted teeth through mechanical dissociation. With the use of minimally invasive surgical technique, the test sites were packed with micrografts seeded onto collagen sponge, whereas the control sites were filled with collagen sponge alone. Their findings exhibited a significantly more probing depth reduction, clinical attachment level gain and bone defect fill in the test sites compared to control ones. It was found that application of DPSCs significantly improved clinical parameters of periodontal regeneration at 1 year after treatment. In addition, Aimetti *et al*[35] performed a case series with 1-year follow-up for 11 patients with chronic periodontitis superimposed by 11 isolated intrabony defects to explore the potential clinical benefits of the application of DPSCs in the regenerative treatment of deep intrabony defects. Extraction was performed for malposed/impacted teeth to be used as autologous source for DPSCs. The defects were accessed with a minimally invasive flap and filled with DPSCs loaded on a collagen sponge. After 1 year posttreatment, a clinical attachment level gain associated with probing depth reduction and a notable stability of the gingival margin was detected using clinical outcomes supported by the radiographic analysis. In 63.6% of the experimental sites, complete pocket closure was achieved. Moreover, a quasi-experimental study was conducted by Hernández-Monjaraz *et al*[36] for 22 adults with PDs, without uncontrolled systemic chronic diseases. Every participant underwent clinical and radiological evaluations and saliva samples were taken to determine the total concentration of interleukins (ILs), antioxidants, lipoperoxides, and superoxide dismutase (SOD). All subjects underwent curettage and periodontal surgery and then two groups were allocated randomly, namely experimental and control groups. The defects of the control group had a collagen scaffold, while the experimental one had a collagen scaffold treated with DPSCs. The experimental group showed an increase in the bone mineral density of the alveolar bone with a borderline statistical significance. Regarding inflammation and oxidative stress markers, IL1β levels had a level decrease, while salivary SOD level was significantly higher in the experimental group. Their findings suggest that treatment based on DPSCs has both an effect on bone regeneration linked to an increased SOD and decreased levels of IL1β in subjects with PDs.

DPSCs-derived from inflammatory pulp: In a scaffold-based trial, Li *et al*[33] harvested DPSCs-IPs from 2 patients with periodontal intrabony defects and then they were loaded onto the scaffold material β-TCP and engrafted into the PD area in the root furcation. At 9 mo after surgical reconstruction, DPSCs-IPs were able to engraft and had an effect of regeneration of new bones to repair PDs. The success rate of primary cell culture and growth status was marginally inhibited; however, DPSCs-IPs expressed comparable levels of stem cell markers as well as retaining their ability to multi-differentiate.

REGENERATION OF SGS USING DPSCS

The parotid, submandibular (SMG) and sublingual glands are the three pairs of major SGs. The anatomical architecture of all three glands is virtually identical, with different ratios of serous and mucous cells: an arborized ductal system that opens into the oral cavity with secretory end pieces, or the acini-producing saliva. The acinar cells are surrounded by an extracellular matrix, myoepithelial cells, myofibroblasts, immune cells, endothelial cells, stromal cells and nerve fibers. The ducts transport and modify the saliva before it is excreted into the oral cavity through the excretory duct[37]. The aim of regenerative medicine is to replace/regenerate the SG epithelium and restore its secretory function in patients suffering from permanent loss of major SG function, after radiotherapy of head and neck cancer, which is the sixth most common cancer in the world and affects tens of thousands of new patients every year, causing hyposalivation/xerostomia with subsequent difficulties in mastication, swallowing, taste, speech, high patient discomfort and a reduced quality of life. Palliative therapies show a limited effectiveness in relieving the symptoms, such as mechanical stimulation of SG activity using chewing gum, or pharmacological agents such as pilocarpine, as well as saliva substitutes[38]. Adult stem cell transplantation strategies have recently shown to enhance clinical outcomes in radiotherapy-induced xerostomia in rodents. Mesenchymal stem cells from adipose tissue are the most promising[39], while those from the labial mucosa, bone marrow or DP have an attractive therapeutic value

following successful findings in *ex vivo* and *in vivo* mouse models of SG injury. DPSCs are supposed to be a good source for SG regeneration by being a component of epithelial acinar cells itself or by being a SG mesenchymal component to encourage and support the regeneration process of the SGs. In SG bioengineering, the principal role of DPSCs is to regenerate the salivary stroma or mesenchymal-derived compartment, and these cells are one of the best choices for that purpose because the tooth mesenchyme and the SG mesenchyme share a common neural crest embryonic origin[40]. A radiation model was developed to reproduce the hyposalivation induced by head and neck cancer therapy. The ductal ligation model aimed to mimic the obstructive sialadenitis and sialithiasis-induced sialopathy and tissue punch considered as a model of mechanical injury (Figure 3). Systemic disease/medication was devised to correspond to Sjogren's syndrome[41].

Preclinical studies

Autologous[42,43], xenogeneic[44] DPSCs or their Exo[45] have been isolated from healthy[42-45] DP. The strategy of using DPSCs was dependent on scaffold-based[42], scaffold-free[43,44] and cell-free[45] therapies. Application of DPSCs was in the form of cell injection[42-44]. Mice were the most selected model for studying the regenerative effect of DPSCs for SG regeneration in the pre-clinical trials. Other animals such as rat[43] were also selected for the same purpose. Irradiation-induced SG hypofunction[43] and duct ligation-induced atrophy[45] or salivary hypofunction induced by diabetes[44] were the presented study models. The cell number of 1×10^6 was the most commonly used[42,43] but 5×10^5[44] cell number was also used, and 0.4 mL[45] of DPSCs-CM was indicated for the cell-free therapy strategy (Table 3).

DPSCs-derived from healthy pulp: (1) Subcutaneous and intraglandular injection of cell suspension of DPSCs. In a scaffold-based trial, Janebodin *et al*[42] created an *in-vivo* model to study the differentiation effect of DPSCs to support SG tissue formation. They subcutaneously transplanted either 1×10^6 hSG cells alone or 1×10^6 hSG cells combined with 1×10^6 murine DPSCs. These cells were transplanted with hyaluronic acid (HA) hydrogel scaffold into 2-mo-old male Rag1 null mice to avoid immune rejection against hSG. The cells were injected ventrally onto SMG without penetrating the gland in the form of 100 μL of cell suspension. After 2 wk of transplantation, HA plugs were dissected without involving mouse recipients' SG tissues, to be investigated with hematoxylin and eosin, periodic acid Schiff and immunofluore-scence staining. Histological evaluation revealed that combination between hSG + DPSCs contributed to the generation of salisphere-like structures. These salispheres expressed markers of terminally differentiated acinar cells and after subcutaneous transplantation in mice, developing a well-irrigated SG-like tissue. These findings provide evidence of the potential use of DPSCs as ecto-mesenchyme for induction and support of SG development.

In a scaffold-free trial, Yamamura *et al*[43] established a mouse model in which DPSCs were used as a cell source for treatment of irradiation-induced SG hypofunction. DPSCs were isolated from female C57BL/6J green fluorescent protein-expressing mice and were differentiated into dental pulp endothelial cells (DPECs). Both SMG of the irradiated mice were injected with 1×10^6 DPECs/gland. Eight weeks after irradiation, the effect of DPECs on saliva secretion was evaluated by measuring amounts of saliva secretion. It was found that transplantation of DPSCs are highly effective in maintaining saliva production after irradiation through direct differentiation of into salivary epithelial cells. This study established a mouse model in which DPSCs could be used as a cell source for the treatment of SG hypofunction. Moreover, Narmada *et al*[44] injected intraglandular tissues of the SMG in diabetic Wistar rat models once with 5×10^5 cells of hDPSCs/250 g in 0.2 mL phosphate-buffered saline solution. The control diabetic rats were injected only with 0.2 mL of phosphate-buffered saline intraglandular. Half of the rats was sacrificed after 7 d, while the remaining rats were sacrificed after 14 d for immunohistochemical evaluation. It was found that transplantation of DPSCs was able to regenerate SMG defects in diabetic Wistar rats by decreasing acinar cell vacuolization.

(2) Secretome from DPSCs. Takeuchi *et al*[45] collected cell culture supernatant from subconfluently DPSCs culture (DPSC-CM). DP tissues were collected from healthy teeth extracted from individuals. The SMG duct was ligated approximately 2 mm superior to the main body of the gland with a sugita titanium aneurysm clip. The experimental groups were divided into a DMEM group (in which 0.4 mL DMEM was administered *via* the right jugular vein biweekly) and a CM group (in which 0.4 mL DPSC-CM was administered using a similar method); the control group was composed of mice that underwent only the release of ligation. Injection of DPSCs-CM

Table 3 Preclinical studies obtained from the PubMed, Scopus and Google Scholar databases related to salivary gland regeneration using dental pulp stem cells

Ref.	Strategy	Model	Defect/site of cell implantation	Cell number	Cell source	Cell application
Janebodin *et al*[42], 2012	Scaffold-based	Rag1 null mice	Injected ventrally onto SMG without penetrating the gland	1×10^6	Murine DPSCs	100 µL of cell suspension
Yamamura *et al*[43], 2013	Scaffold-free	C57BL/6J green fluorescent protein-expressing mice	Irradiated SMG	2×10^6	Murine DPSCs	5 µL of single cell suspension was injected intraglandular using a 28 G needle and microliter syringe.
Narmada *et al*[44], 2020	Scaffold-free	Wistar rats	SMG of diabetic rat model	5×10^5	hDPSCs	Intraglandular cell injection.
Takeuchi *et al*[45], 2020	Cell-free	ICR mice	SMG duct ligation	0.4 mL	hDPSCs	DPSCs-CM was administered *via* the right jugular vein biweekly under general anaesthesia.

DPSCs: Dental pulp stem cells; hDPSCs: Human dental pulp stem cells; SMG: Submandibular.

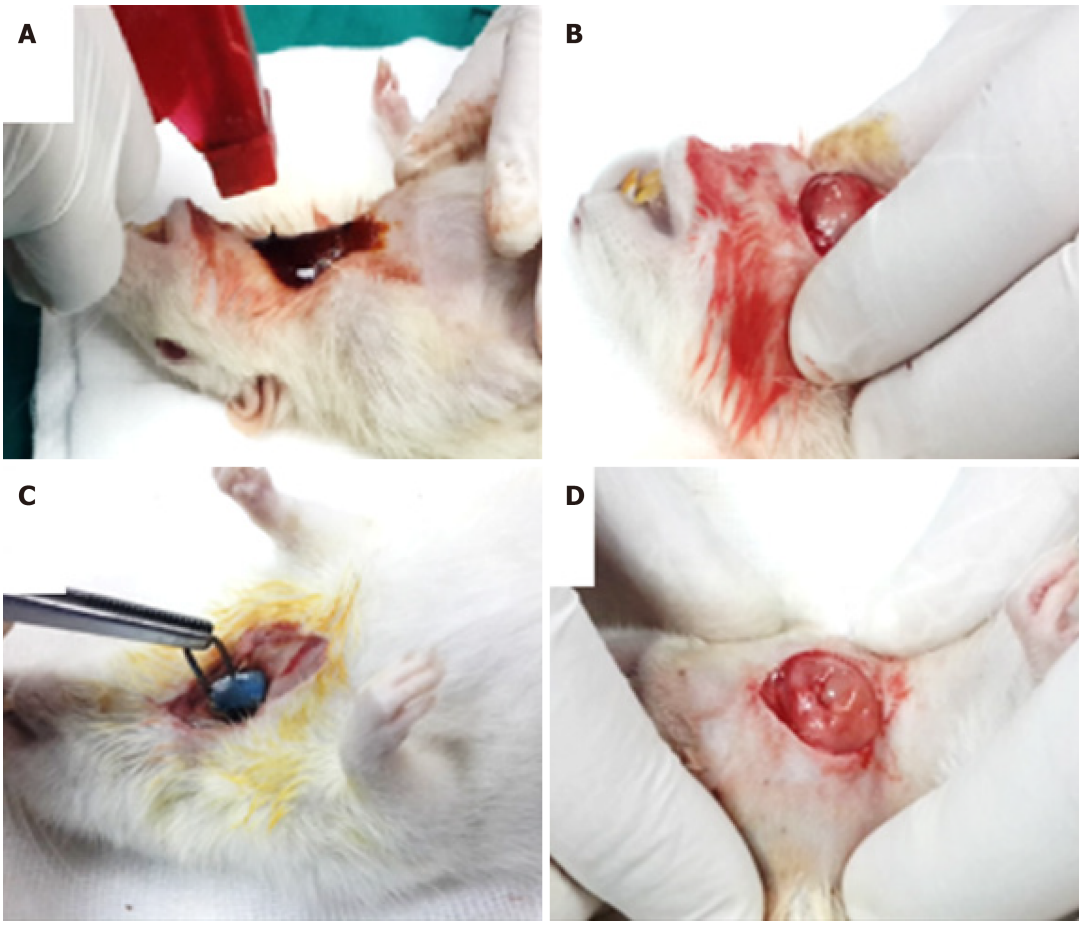


Figure 3 Punch biopsy of submandibular gland. A: Photographs showing a tissue punch of submandibular (SMG) as a model of mechanical injury through disinfecting the skin of operation site by betadine; B: Exposing the right SMG; C: Applying the tissue punch on the center of right SMG; D: The arrow points to a rectangular cut area of 5 mm × 8 mm × 1.5 mm dimensions. Courtesy provided by the staff members of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.

to mice after releasing the SMG main duct ligation significantly elevated the expression levels of precursor cell marker CK5 and acinar cell marker AQP5 after the release. Growth factors contained in DPSCs-CM were considered to be responsible for the effects. This study suggested that DPSCs-CM administration to mice after releasing the SMG main duct ligation may promote acinar cell regeneration of atrophied SGs.

REGENERATION OF BONE DEFECTS USING DPSCS

Bone fractures appear to occur more often in old age because of bone weakening due to a reduction in calcium content in extracellular matrix and slowing of bone remodeling processes. Current methods of treatment are unable to fully reconstruct bone defects that are caused by fractures, tumors and congenital abnormalities. At present, autogenous or allogeneic bones and artificial materials are used for bone augmentation. However, there are various problems with these grafts, such as the possibility of infection or absorption as well as ethical issues. Alternatively, the periosteum and the bone marrow are considered rich in bone progenitors, so that the bone, since early 1990s, has been one of the approachable goals of clinical TE[46]. In addition, osteogenic differentiation of DPSCs is easily induced *in vitro* by adding ascorbic acid, dexamethasone, and β-glycerophosphate to the culture medium, along with fetal bovine serum, making these cells a promising tool for bone regeneration[47]. Bone defect repair is characterized by three overlapping processes, namely the formation of blood clot, bone formation, and bone remodeling. Osteoprogenitor cells migrate to the wound site during the second phase, proliferate and differentiate into osteoblasts that segregate and induce mineralization. DPSCs are a vital source of osteoprogenitor cells which play a fundamental role in the bone tissue plasticity-related mechanisms[48-50].

Preclinical studies

Autologous[51] or xenogeneic[49,50,52] DPSCs have been isolated from healthy[50-52] DP. The strategy of using DPSCs was dependent on scaffold-based[52], scaffold-free[50,51] therapies. Application of DPSCs was in the form of cell sheet transplantation[51], loaded onto the scaffold[52] or, incorporated onto the PRP and the combination was injected locally[50]. Animals such as rabbit[52], mice[51] and dogs[50] were selected to study bone regeneration preclinically. Mandibular[50] (Figure 4), calvarial[51] and craniofacial[52] bone defects were presented. The cell number of 1×10^7 was the most commonly implicated[50] but 2×10^6[52] was also used (Table 4).

DPSCs-derived from healthy pulp: (1) Loading or injecting a cell suspension of DPSCs. In a scaffold-based trial, Mohammed *et al*[52] loaded 2×10^6 hDPSCs on gel foam scaffold to be fitted in induced temporomandibular joint defect area of 2.5 mm × 2.5 mm in diameter and in depth for the rabbit's mandible. The histological investigation was performed for qualitative evaluation of bone healing and it was found that newly formed bone with thick trabecular pattern and less osteoid tissue have been observed in the experimental rabbits transplanted with osteogenic-differentiated hDPSCs. The authors suggested the ability of transplanted osteogenic differentiated hDPSCs to produce new osteoid tissue that helps in repairing an induced rabbit mandibular defect in a time and differentiation phase-dependent manner. In an attempt to confirm that bone regeneration was indeed the function of hDPSCs, Chamieh *et al*[53] tested the efficacy of DPSCs seeded on a collagen scaffold for craniofacial bone regeneration. Plastically compressed collagen gels were loaded with DPSCs (2×10^6 cells/mL) which were isolated from molar Wistar rats. A 5-mm diameter calvarial critical-sized defect was created on each side of the parietal bone using a dental bur attached to a slow-speed hand piece operating at 1500 rpm, under irrigation with sterile saline solution; empty defects were created as a negative control for the critical-sized defects. It was found that collagen gel scaffolds seeded with DPSCs improved bone healing and the remodeling process under micro-X-ray computed tomography as well as histological examination.

Liu *et al*[50] utilized nano-hydroxyapatite/collagen/PLLA as a scaffold for autologous DPSCs seeding to reconstruct critical-size alveolar bone defects in New Zealand rabbit. A 10 mm incision was made and the tissue overlying the diaphysis of the left alveolar bone of incisors of rabbits was dissected. A segmental defect of 10 mm × 4 mm × 3 mm was prepared in the alveolar bone. Cell number of 1×10^6 DPSCs was implanted onto the scaffold and transplanted into the alveolar bone defect. The animals were sacrificed 12 wk after operation for histological observation and histomorphometric analysis. It was found that reconstruction of alveolar bone defects using DPSCs seeded on nano-hydroxyapatite/collagen/PLLA had earlier mineralization and more bone formation than achieved with the scaffold alone or even the autologous bone graft.

In a scaffold-free trial, Yamada *et al*[49] tried to test the osseointegration around the implant during the healing period using DPSCs. Human DPSCs were obtained from extracted permanent teeth. Adult hybrid dogs with a mean age of 2 years were

Table 4 Preclinical and clinical studies obtained from the PubMed, Scopus and Google Scholar databases related to bone regeneration using dental pulp stem cells

Ref.	Strategy	Model	Defect/site of cell implantation	Cell number	Cell source	Cell application
Yamada *et al*[49], 2010	Scaffold-free	Dogs	Mandibular bone defects were prepared using a trephine bur with a diameter of 10 mm	1×10^7	hDPSCs	Cavities filled with DPSCs/PRP
Liu *et al*[50], 2011	Scaffold-based	Rabbit	A segmental critical size defect (10 mm × 4 mm × 3 mm) was prepared in the alveolar bone	1×10^6	Autologous DPSCs	DPSCs were seeded onto nano-hydroxyapatite/collagen/PLLA and implanted *in-vivo*
Fujii *et al*[51], 2018	Scaffold-free	Mice	3.5 mm calvarial defects	NA	Autologous DPSCs	Cell sheet transplantation
Mohammed *et al*[52], 2019	Scaffold-based	Rabbit	Craniofacial bone defect in the left side of TMJ	2×10^6	hDPSCs	DPSCs were loaded on gel foam
Chamieh *et al*[53], 2019	Scaffold-based	Rat	Non-critical defects of the tibia in rats	10^6 cells/mL	hDPSCs	Loaded on a lyophilized and hydrolyzed collagen sponge (Hemospon®)
d'Aquino *et al*[54], 2009	Scaffold-based	Human	Defect without walls, of at least 1.5 cm in height	1×10^7	Autologous DPSCs	Cells endorsed with a syringe onto collagen sponge scaffold

DPSCs: Dental pulp stem cells; hDPSCs: Human dental pulp stem cells; NA: Not available; PRP: Platelet-rich plasma.

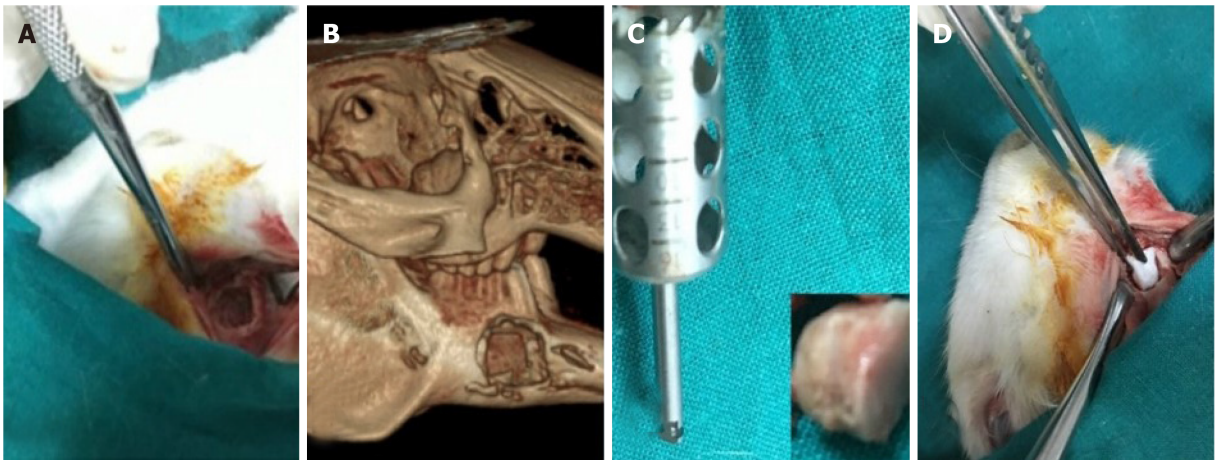


Figure 4 Mandibular bone defect. A: Photograph showing a 10-mm diameter, full-thickness mandibular defect with the removal of the mandibular first premolar and both cortical plates; B: Cone-beam computed tomography systems reconstruction of the critical-size bone defect; C: The 10-mm trephine bur with autogenous tibial bone graft; D: Gelfoam placed on the defect. Courtesy provided by the staff members of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.

operated on under general anesthesia. In the mandible region, the first molar and the second and third premolars were extracted, and the healing period was 2 mo. Bone defects were prepared on both sides of the mandible using a trephine bur with a diameter of 10 mm. The defects were implanted with DPSCs or BMSCs (1×10^7 cell/mL) plus PRP while the defect-only represented the control group. After 8 wk, an HA-coated osseointegrated dental implant was inserted into the bone regeneration areas and was assessed histologically. The dogs were sacrificed 16 wk after insertion of the dental implant. A histological analysis was performed to obtain a general description of the tissue surrounding the implants. Cavities filled with DPSCs/PRP or BMSCs/PRP were found to show new bone formation at 8 wk with a tubular pattern along with abundant vascularization. This pattern reflected normal bone macrostructure with well-differentiated marrow cavity and cortices, while the cavities in the control group were invaded by vascular and fibrous tissues. Bone-implant contact revealed significant increase in the group treated with DPSCs/PRP or BMSCs/PRP in comparison to controls.

(2) Cell sheet transplantation technique of DPSCs. In a scaffold-free trial, Fujii *et al*[51] evaluated bone regeneration by hDPSCs using a helioxanthin derivative and cell-sheet technology. DPSCs were cultured on temperature-responsive dishes after

treatment with osteogenic medium for 14 d then the sheets were folded to fit the 3.5-mm calvarial defects of mice. The skin and subcutaneous layer were then closed by 6-0 nylon sutures. Eight weeks after surgery, calvarial defect sites were harvested from euthanized mice. Micro-CT images showed that the DPSC sheets were able to induce bone formation without requiring scaffold or growth factors. Cell sheets are flexible and simple to transplant, so this technology may replace conventional bone graft methods in the future.

Clinical studies

Autologous hDPSCs were the source for a RCT[42] with 3 mo follow-up case series. The strategy of using DPSCs was dependent on scaffold-based only. Patients diagnosed with bilateral bone resorption following impaction surgery of impacted third molars are presented (Table 4).

DPSCs for cell and scaffold-based strategy: D'Aquino *et al*[54] utilized DPSCs and a collagen sponge scaffold for oro-maxillo-facial bone tissue repair in patients requiring extraction of their third molars. The patients suffered from bilateral alveolar bone reabsorption distal to the second molar following impaction of the third molar on the cortical alveolar lamina, producing a defect without walls, of 1.5 cm in height. Extraction of the third molar in this clinical condition does not permit spontaneous bone repair and ultimately leads to loss of the adjacent second molar as well. For DPC isolation and expansion, maxillary third molars were extracted. The cells were then seeded onto a collagen sponge scaffold and the biocomplex obtained was used to fill in the injury site left by extraction of the mandibular third molars. At 3 mo after autologous DPSCs grafting, the patients alveolar bone had optimal vertical repair and complete restoration of periodontal tissue back to the second molars, as assessed by clinical probing and X-rays.

CONCLUSION

DPSCs have emerged as a promising tool with a great potential for dental and oral tissue regeneration or engineering. However, many issues and challenges still need to be addressed before these cells can be employed in clinical practice. The full control of differentiation of DPSCs to specific fates is still an important issue, and although DPSCs derivation to certain connective tissue-lineage cells appears to be relatively simple, their differentiation into nerve-tissue lineages still poses important unanswered questions. Moreover, the development of the cell recombination technology required to create next-generation bioengineered replacement organs will necessitate extensive research for the coming years. In this case, DPSCs may be an ideal choice to be used in experimental models of tissue-engineered reconstruction of organs of the oral cavity, and the paradigmatic cases of the tooth and the SG may constitute the leading edge of a massive research effort on next generation organ replacement therapies[2].

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 April 28; 9(2): 193-207

DOI: 10.13105/wjma.v9.i2.193

ISSN 2308-3840 (online)

SYSTEMATIC REVIEWS

Laboratory hematologic features of COVID-19 associated liver injury: A systematic review

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Author contributions: Frater JL designed the research study, performed the research, analyzed the data, and wrote the manuscript; Wang T and Lee YS analyzed the data; All authors have read and approved the final manuscript.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

PRISMA 2009 Checklist statement: The manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Liver injury is a common complication of infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. The utility of laboratory hematology data in the diagnosis and risk stratification of patients with coronavirus disease 2019 (COVID-19) has not been comprehensively examined.

AIM

To address the following. (1) Are the abnormalities in hematologic parameters seen in the general population of patients with COVID-19 also seen in those patients with associated liver injury? (2) Is liver injury in COVID-19 a sign of severe disease and does liver injury correlate with hematologic markers of severe disease? And (3) What is the quality of this evidence?

METHODS

To address these questions, a comprehensive systematic review was performed. We searched the peer reviewed medical literature using MEDLINE (PubMed interface), Web of Science, and EMBASE for cohort studies that specifically addressed liver injury and COVID-19 without limitation of date of publication or language. A quality assessment of the studies was performed using the Newcastle-Ottawa Scale.

RESULTS

Thirty-two articles were suitable for inclusion in our systematic review. These included 22 articles with a cohort of COVID-19 patients with liver injury, 5 comparing non-severe *vs* severe COVID-19 populations in which liver injury was addressed, and 5 other cohort studies with a focus on liver injury. White blood cell count, absolute neutrophil count, absolute lymphocyte count (ALC), and hemoglobin were the parameters most helpful in distinguishing COVID-19 with liver injury from COVID-19 without liver injury. ALC and d-dimer were identified as being potentially useful in distinguishing non-severe from severe

Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E

Received: January 28, 2021
Peer-review started: January 28, 2021
First decision: February 24, 2021
Revised: March 31, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Braga MB, Duan Z
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li JH

COVID-19. Liver injury was more frequently seen in cohorts with severe disease. Most studies were of high quality (24/48, 86%) with 4/28 (14%) of moderate quality and 0 of low quality.

CONCLUSION

Our study supports the use of select hematologic parameters in diagnosis and risk stratification of liver injury in COVID-19 patients. Although of overall high quality, the current medical literature is limited by the small number of studies with high statistical power and the variable definition of COVID-19 liver injury in the literature.

Key Words: COVID-19; SARS-CoV-2; Laboratory hematology; Systematic review; Coagulation; Liver injury

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Core Tip: The use of laboratory hematology data as biomarkers of disease has not been examined for coronavirus disease 2019 (COVID-19) patients with liver injury, and the clinical significance of liver injury in COVID-19 is unclear. By means of a systematic review we showed that blood cell count, absolute neutrophil count, absolute lymphocyte count (ALC), and hemoglobin are of potential use in identifying liver failure in COVID-19. ALC and d-dimer have potential utility in distinguishing severe from non-severe disease in patients with liver injury. Our findings provide a rationale for further studies with sufficiently large numbers of patients and rigorous definition of liver injury to validate these findings.

Citation: Frater JL, Wang T, Lee YS. Laboratory hematologic features of COVID-19 associated liver injury: A systematic review. *World J Meta-Anal* 2021; 9(2): 193-207
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/193.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.193

INTRODUCTION

Rationale

The coronavirus disease 2019 (COVID-19) pandemic, caused by infection by the beta coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been the subject of a vast number of studies in the medical literature; a January 16, 2021 search of the MEDLINE database (PubMed interface) for "COVID-19" identified 92967 results. A recent scoping review of the laboratory hematology literature identified > 400 studies, of which a high fraction were related to the roles of automated hematology, coagulation, and flow cytometry for diagnosis, prognosis, and risk stratification in COVID-19[1]. As a result of this work, several abnormalities of complete blood count (CBC) and coagulation testing have been identified as biomarkers of adverse risk in infected patients. These include leukocytosis[2], lymphopenia[2-4], thrombocytopenia[2,5], neutrophilia[2,3,6-9], prolonged prothro-mbin time (PT) and activated partial thromboplastin time (aPTT)[2], and elevated d-dimer[1,2]. It is unclear whether this information can be applied to specific subpopulations of patients with COVID-19 disease, such as those with liver injury[10].

Liver injury in COVID-19 has been defined as "any liver damage occurring during disease progression and treatment of SARS-CoV-2 infection"[11]. The classification of liver injury in COVID-19 is not systematized due to a lack of international consensus[12-15]. Elevated biochemical liver enzymes including aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin (BILI) are a common finding in COVID-19 infection[2,4,6,16,17] and have been reported in approximately 50% of all COVID-19 patients and approximately 75% of hospitalized patients at admission[18-23]. COVID-19-related liver injury has been variously defined by increased levels of AST, AST, gamma-glutamyltransferase (GGT), lactate dehydrogenase, alkaline phosphatase (ALP), or total bilirubin (T-BIL) above the upper limit of normal (ULN)[13,19,21,24-28]. Whether the elevation of liver enzymes in COVID-19

patients reflects liver injury in all cases is controversial[29], although a study by Bloom *et al*[30] concluded that elevated liver enzymes in hospitalized patients appeared to be related to true liver injury. The mechanism of liver injury is unclear and may be due to the direct effects of the virus, drug effect, the impact of systemic inflammation, microthrombosis, or some combination of factors[21,31-37]. The extent to which COVID-19-associated liver injury contributes to morbidity and mortality is controversial, although the current literature suggests that higher levels of liver enzymes correlate with more severe liver injury[16,38-42].

Objectives

The purpose of this study was to assess the medical literature to answer the following questions. (1) To what extent are previously reported hematologic abnormalities seen in patients with COVID-19-associated liver injury? (2) Based on the combination of outcome data and hematologic findings, is liver injury in COVID-19 a sign of severe disease? Does liver injury correlate with markers of severe disease? And (3) What is the quality of this evidence? These questions are significant, since CBC and coagulation testing are among the most commonly ordered clinical laboratory tests, provide rapid results, and can be performed in virtually any setting, including resource-limited environments in which SARS-CoV-2 testing is unavailable. This is a systematic review that uses specific evidence-based criteria based on "explicit, prespecified, and reproducible methods"[43] to demonstrate where knowledge is lacking and provide guidance for future research[44].

MATERIALS AND METHODS

Format

This study was performed using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist.

Information sources and search strategy

We conducted an electronic search of Medline (PubMed interface), EMBASE, and Web of Science using the keywords "COVID-19" and synonyms ("SARS-CoV-2," "2019 novel coronavirus," "2019-nCoV," "Wuhan coronavirus," "novel coronavirus," "Wuhan seafood market pneumonia virus," "Wuhan virus") AND "liver injury." The search was performed performed on January 13, 2021 without date limitations. This protocol has not been registered. Individual authors were not contacted.

Eligibility criteria

We included the COVID-19 literature related to liver injury indexed by January 13, 2021. There was no restriction of article type in the search; restrictions of article type were applied during the screening process to avoid inadvertently missing relevant articles.

Data collection process

Titles and abstracts of all articles were screened. Following initial screening, the full text of all studies considered for potential inclusion in the analyses were reviewed for suitability. For articles published in Chinese, the full text was evaluated by a member of the research team fluent in that language (Lee YS). Then reviews, meta-analyses, editorials and other opinion pieces, and articles not focused on the study topic were removed. The full text of the articles was reviewed, resulting in another group of papers being eliminated. This resulted in a final group of articles remaining for the qualitative analyses, which included: Studies with a COVID-19 (+) cohort with liver injury provided as a distinct cohort; studies that compared patients with severe *vs* non-severe COVID-19 disease, in which information about liver injury in these groups was provided; and papers with other study designs that included information regarding liver injury in COVID-19 disease. The content of the included studies was critically appraised by the authors, who are experts in the field of pathology/laboratory hematology (Lee YS, Frater JL) or clinical science (Wang T).

Synthesis of results

Extracted data and descriptive statistics were entered and performed using Excel (Microsoft, Redmond, WA, United States). For studies in which a COVID-19 (+) cohort with liver injury provided as a distinct group, the following data were extracted:

Authors; date of publication; country where study was performed; number of patients in COVID-19 (+) cohort with liver injury; percentage of male patients in COVID-19, (+) cohort with liver injury; setting in which the study was performed; criteria for liver injury used by the study; presence of, and criteria for inclusion in the control group; hematologic parameters evaluated in the study; and results of studies of statistical significance of each hematologic parameter. For studies that compared patients with severe *vs* non-severe COVID-19 disease, the following information was extracted: Authors, date of publication, country where study was performed, number of patients in the COVID-19 (+) cohort with liver injury, setting in which the study was performed, criteria used by the study to categorize patients as having severe disease, criteria for liver injury used by the study, hematologic parameters evaluated in the study, and summary of the main findings as they pertain to the study topic. For papers with other study designs, the following information was extracted: Authors, date of publication, country where study was performed, setting in which the study was performed, hematologic parameters evaluated in the study, description of study design, and summary of the main findings as they pertain to the study topic.

Additional analyses (quality assessment)

Two authors (Wang T and Frater JL) assessed the study quality of the cohort studies using the Newcastle-Ottawa Scale (NOS)[45]. We designated the quality of each study as high (7-9 points), moderate (4-6 points), or low (0-3 points). In situations where the reviewers disagreed on a rating, a third reviewer (Lee YS) adjudicated and provided consensus.

RESULTS

Study selection

Through a search of the databases, we identified a total of 967 potential articles. Following the removal duplicate articles, we screened the titles and abstracts of the remaining articles to further identify potentially relevant articles. After thorough assessment of all full-text articles, 32 studies met the established inclusion criteria and were included in this systematic review (Figure 1).

Study characteristics

A total of 22 studies had a COVID-19 (+) cohort with liver injury provided as a distinct group (Table 1)[26,46-66].

All studies were retrospective and included nine studies from China, five studies from the United States, two studies from Japan, two studies from Iran, and one study each from Germany, Portugal, France, and Italy. The median number of patients in the liver injury cohort of each study varied from 2 to 1784 (median 73.5 patients). A male predominance was identified in all but four studies. In 19 studies, the COVID-19(+) with liver injury group was presented as a single cohort. Inclusion criteria for the study groups were variable. In 15 studies, all patients with a combination of AST, ALT, BILI, and GGT exceeding the ULN were categorized in the liver injury group. In one study, the liver injury group was limited to patients with ALT ≥ 3 times the ULN[53]. In the remaining studies, the COVID-19(+) with liver injury groups were stratified into two[48,55] or three groups[60] based on serum liver enzyme levels. In one study[54], a study reporting the results of liver biopsy in two COVID-19 patients with liver involvement and no evidence of pulmonary disease, inclusion in the study was based on the results of the liver biopsies. In another study[56], an autopsy-based report of 11 patients, inclusion was based on positive RT-PCR testing of liver tissue for SARS-Cov-2. The average age of the study group ranged from 41-years-old to 70-years-old. In all but three studies a male predominance was seen in the liver injury group. Most studies drew their study group from hospitalized inpatients, one study was conducted with critical care patients, and two studies were conducted with non-critical care inpatients.

Eighteen studies (4973 patients) in this group contained statistical data that allowed comparative analyses of the following hematologic parameters: White blood cell count (WBC), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), hemoglobin (HGB), platelet (PLT) count, d-dimer (DDIM), PT, PTT, and international normalized ratio (INR) (Table 2).

Although a direct comparison of the studies is limited by several factors such as heterogenous inclusion criteria and variable sample sizes, the majority of studies in which the inclusion criteria for liver injury were liver enzyme levels > ULN have demonstrated statistically significant differences in WBC, ANC, and ALC between

Table 1 Studies with a coronavirus disease 2019 (+) cohort with liver injury provided as a distinct group

Ref.	Country	Number of patients	% of total patients	Age, mean	% male	Setting	Study group inclusion criteria	Control group	Evaluated parameters	NOS
Abe *et al*[46]	Japan	15	68	47	59	Hospitalized patients	ALT OR GGT over ULN	COVID+	WBC, ALC, HGB	8
An *et al*[47]	China	51	21	52	45.1	Hospitalized patients	AST OR ALT over ULN	COVID+	WBC, ALC, ANC, DDIM	8
Anastasiou *et al*[48]	Germany	82	56.2	64.5	74	Hospitalized patients	ALT OR BILI over ULN	COVID+	WBC, INR	6
		11	6.8	51	100		EASL ALF criteria (see ref[10])			
Cardoso *et al*[49]	Portugal	20	100	67	90	ICU	GGT OR/AST OR/ALT over ULN	None	SOFA, APACHE II	NA
Chen *et al*[50]	China	227	38	56	63	Hospitalized patients	ALT OR AST OR GGT over ULN	COVID+	ALC, DDIM	9
Chen *et al*[51]	China	301	59	62	82	Hospitalized patients	AST OR ALT OVER ULN/ OR elevated ALP/GGT over ULN	COVID+	WBC, ALC, ANC, PLTs, PT, PTT, INR	7
Chu *et al*[52]	China	429	51	61	65	Hospitalized patients	ALT/ALP ratio over ULN	COVID+	WBC, ALC, ANC, PLTs, HGB, DDIM, PT, INR	7
Da *et al*[53]	U.S.	109	62	61	78	Hospitalized patients	AST or ALT ≥ 3 times ULN	COVID+	DDIM, PT, INR	8
Fiel *et al*[54]	United States	2	100	NA	50	Hospitalized patients	Biopsy proven disease without pneumonia	None	DDIM	NA
Huang *et al*[55]	China	253	37.5	58	59	Hospitalized patients	AST, ALT, TBIL over ULN	COVID+	WBC, ALC, PLTs, HGB	5
		52	7.7	51.5	81		ALT ≥ 3 times ULN			
Lagana *et al*[56]	United States	11	100	70	70	NS	PCR testing of liver	None	DDIM	NA
Li *et al*[57]	China	7	NS	61	43	Hospitalized patients	AST or ALT over ULN	None	WBC, ALC, ANC, DDIM, HGB	NA
Meszaros *et al*[58]	France	156	66.7	66.5	73	Hospitalized patients	AST OR ALT OR GGT over ULN	COVID+	ALC, PLTs, DDIM	7
Mishra *et al*[59]	United States	184	52.9	62.7	58	Hospitalized patients	AST OR ALT over ULN	COVID+	WBC, ALC, ANC, HGB	7
Phipps *et al*[60]	United States	1784		66	73	Hospitalized patients	ALT > 1-2× ULN	COVID+	WBC, ALC, ANC, PLTs, DDIM, HGB	8
		344		61			ALT 2-5× ULN			
		145		63			ALT > 5× ULN			
Piano *et al*[61]	Italy	329	58	66	63	Hospitalized patients	AST OR ALT OR GGT OR ALP OR BILI > ULN	COVID+	WBC, ALC, ANC, PLTs, DDIM, INR	7
Qi *et al*[62]	China	32	46	41	72	Hospitalized patients	AST OR ALT OR BILI over ULN	COVID+	WBC, ALC, ANC	6

Sadeghi *et al*[64]	Iran	65	64	55	64	Non-ICU hospitalized	AST OR ALT OR BILI over ULN	COVID+	WBC, ALC, ANC	9
Safari *et al*[63]	Iran	41	17	52	31	Hospitalized patients	ALT above ULN	COVID+	WBC, PLTs, HGB	8
Tsutsumi *et al*[65]	Japan	31	52	46	84	Hospitalized patients	ALT ≥ 40 U/L	COVID+	WBC, ALC, PLTs, DDIM, PT	8
Wang *et al*[66]	China	303	46.1	62	63.4	Hospitalized patients	ALT OR GGT OR BILI over ULN	COVID+	WBC, ALC, ANC, PLTs, HGB, PT	8
Xie *et al*[26]	China	29	26.9	62	72.4	Non-ICU hospitalized	AST OR ALT OR BILI over ULN	COVID+	WBC, ALC, ANC, DDIM	8

ALC: Absolute lymphocyte count; ALP: Alkaline phosphatase; ALT: Alanine transaminase; ANC: Absolute neutrophil count; APACHE II: Acute Physiology and Chronic Health Evaluation II scoring system; AST: Aspartate transaminase; BILI: Bilirubin; COVID-19: Coronavirus disease 2019; DDIM: D-dimer; EASL ALF criteria: European Association for the Study of the Liver Acute Liver Failure Guidelines; GGT: Gamma-glutamyltransferase; HGB: Hemoglobin; INR: International normalized ratio; NA: Not applicable; NOS: Newcastle-Ottawa Scale; NS: Not stated; PLT: Platelet count; PT: Prothrombin time; PTT: Partial thromboplastin time; SOFA: Sequential Organ Failure Assessment scoring system; ULN: Upper limit of normal; WBC: White blood cell count.

non-liver injury *vs* liver injury groups. Although smaller numbers of studies have addressed patients with higher elevations of liver enzymes, there were still statistically significant differences in WBCs between non-liver injury *vs* liver injury groups. Limiting the analysis to studies with > 100 patients in the high liver function test (LFT) cohort (n = 10 studies, 4564 patients), statistically significant differences in WBC, ANC, ALC, and HGB were identified between non-liver injury *vs* liver injury groups in the majority of studies. These differences appear most pronounced for WBCs and HGB, in which all studies demonstrated statistical significance; and ANC, in which 5/6 studies demonstrated statistical significance.

Five additional studies (425 patients), all retrospective, compared COVID-19 patients with severe *vs* non-severe disease and contained information about liver injury[67-71]. The major features of these studies are summarized in Table 3 and provide some additional context to the information in the liver injury *vs* non-liver injury cohort studies about the potential use of hematology data. These include the study of Liao *et al*[70], which demonstrated that liver injury correlated with inflammatory cytokine level, ALC, and DDIM; the study by Lei *et al*[69], which showed that liver injury correlated with disease severity and ALC; and the studies of Cai *et al*[67] and Jiang *et al*[68], which identified a high percentage of patients with liver failure in the severe disease cohorts, which differed from the non-severe cohorts in ALC and DDIM levels.

Five other retrospective cohort studies (Table 4) likewise demonstrated the potential usefulness of hematology data in COVID-19 disease and liver injury[72-76]. These included the study of Cao *et al*[72] which demonstrated that serum ferritin levels correlated with disease severity and degree of liver injury, as well as showed statistically significant differences in WBC, ANC, ALC, and DDIM between the two groups. Another study correlating elevated liver enzymes/liver injury with increased risk of COVID-19 disease recurrence noted significant differences in PLTs between patients with non-recurrent *vs* recurrent disease[73]. The study of Ding *et al*[74], which associated AST and direct bilirubin elevation with increased risk of mortality also noted significant differences in ALC, PLTs, and DDIM between the two groups. Lei *et al*[75] in a study examining peak levels of AST, ALC, ALP, and TBIL in patients with COVID-19 and liver injury noted that peak levels correlated with ANC, ALC, PLTS, and DDIM. Finally, Luan *et al*[76], who noted the correlation of COVID-19-related coagulopathy with liver injury, also found an associated significant difference in ALC, ANC, and DDIM between patients with and without coagulopathy.

Additional analysis (study quality)

Twenty-eight studies in our series contained control groups and could be evaluated using the NOS for cohort studies. Overall, 24/28 (86%) studies had a NOS in the high quality (7-9) range, 4/28 (14%) in the moderate quality (4-6) range, and no studies were rated in the low quality (0-3) range. Notably, for 24/28 (86%) papers, there was consensus in the initial evaluation by Frater JL and Wang T.

Table 2 Summary of statistical data from studies comparing liver injury *vs* non-liver injury

LFT criteria	Parameter	Significance			
		NS	< 0.05	< 0.01	< 0.001
LFT > ULN	WBC	4	2	0	6
	ANC	4	1	0	4
	ALC	4	2	1	5
	HGB	4	2	0	5
	PLT	4	2	0	2
	DDIM	4	2	1	0
	PT	4	2	1	0
	PTT	4	2	0	0
	INR	4	2	1	0
LFT > 2 × ULN	WBC	4	2	0	3
	ANC	4	2	0	1
	ALC	4	2	1	1
	HGB	4	2	0	2
	PLT	4	2	0	1
	DDIM	4	2	0	1
	PT	4	2	0	0
	INR	4	2	0	0
Studies with > 100 patients in high LFT cohort	WBC	0	0	0	5
	ANC	1	0	0	5
	ALC	4	0	1	5
	HGB	0	0	0	5
	PLT	5	0	0	2
	DDIM	3	0	0	3
	PT	2	1	1	0
	PTT	1	0	0	0

Numbers indicate number of studies. ALC: Absolute lymphocyte count; ANC: Absolute neutrophil count; DDIM: D-dimer; HGB: Hemoglobin; INR: International normalized ratio; LFT: Liver function test; NS: Not significant; PLT: Platelet count; PT: Prothrombin time; PTT: Partial thromboplastin time; ULN: Upper limit of normal; WBC: White blood cell count.

DISCUSSION

Summary of evidence

The purpose of this systematic review was to evaluate whether the hematologic parameters of importance in diagnosis and risk stratification in COVID-19 can be applied to the subpopulation with liver injury, and to determine whether the current state of the evidence supports whether SARS-CoV-2 patients should be categorized as having severe disease. A total of 32 studies met our inclusion criteria and were included in the analyses. The overall quality of the studies was strong, with 86% having an NOS ≥ 7. Based on our evaluation, WBCs, ANC, ALC, and HGB appear to the most useful parameters in addition to LFT to distinguish COVID-19 patients with liver injury from those without liver injury. Similarly, studies that analyzed severe *vs* non-severe COVID-19 cohorts showed that liver injury was present in a high proportion of patients with severe disease, who also had significant differences in ALC and DDIM. Studies focused on liver injury with other study designs likewise supported the association of liver injury with altered WBC, ANC, ALC, PLTs, and

Table 3 Studies comparing coronavirus disease 2019 patients with severe *vs* non-severe disease

Ref.	Country	Patients with severe disease (% of total)	Setting	Definition of SEVERE disease	Definition of liver Injury	Evaluated parameters	Summary of main findings	NOS
Cai *et al*[67]	China	58 (19.5)	Hospitalized patients	Radiography supporting severe disease	ALT OR AST > 3 times ULN or GGT or TBIL > 2 times ULN	WBC, ANC, ALC, DDIM	LFT significantly elevated in severe cohort at admission	8
Jiang *et al*[68]	China	27 (20.6)	Hospitalized patients	Chinese National Health Committee criteria (see ref[82])	AST OR ALT OR ALP OR TBIL > 5 times ULN	WBC, ANC, ALC, PLTS, HGB	Liver injury in 81% of severe cohort over course of hospitalization; slow recovery from liver injury compared to other cohorts	7
Lei *et al*[69]	China	48 Severe cohort	Hospitalized patients	National Health Commission of the People's Republic of China and National Administration of Traditional Chinese Medicine of the People's Republic of China criteria (see ref[83])	CT showing liver hypodensity and pericholecystic fat stranding	WBC, ALC, PLTS	Severity of COVID-19 disease correlated with liver function	7
	China	48 Critical cohort	Hospitalized patients	Health Commission of the People's Republic of China and National Administration of Traditional Chinese Medicine of the People's Republic of China criteria (see ref[83])	CT showing liver hypodensity and pericholecystic fat stranding	WBC, ALC, PLTS	Severity of COVID-19 disease correlated with liver function	
Liao *et al*[70]	China	29 (15.1)	Hospitalized patients	National Guidelines for 2019 novel coronavirus-associated pneumonia (the fifth revised version)	AST OR ALT OR TBIL over ULN	WBC, ANC, ALC, HGB, DDIM	Liver injury in high percentages of patients from both groups; correlated with inflammatory cytokine levels	7
Yang *et al*[71]	China	215 (72.9)	Hospitalized patients	WHO criteria (see ref[8] from[68])	AST > 1.5 times ULN	WBC, ANC, ALC, PLT, DDIM, PT	AST and GGT levels correlated with disease severity	7

ALC: Absolute lymphocyte count; ALT: Alanine transaminase; ANC: Absolute neutrophil count; AST: Aspartate transaminase; COVID-19: Coronavirus disease 2019; CT: Computerized tomography; DDIM: D-dimer; GGT: Gamma-glutamyltransferase; HGB: Hemoglobin; NOS: Newcastle-Ottawa Score; PLT: Platelet count; PT: Prothrombin time; TBIL: Total bilirubin; WBC: White blood cell count; WHO: World Health Organization; ULN: Upper limit of normal.

DDIM.

Association of liver injury with abnormalities of hematologic parameters: Diagnosis and risk stratification

Automated hematology data and basic coagulation testing is widely available, cost-effective, and can be performed with rapid turnaround time. In situations where this data is of proven utility, it can provide value to the clinical team, either alone or as a component of other clinical and laboratory testing predictive models, such as the Sequential Organ Failure Assessment (SOFA) and Acute Physiology and Chronic Health Evaluation II scoring systems, which are widely used in critical care medicine.

Increased WBC has been associated with severe disease in COVID-19, and in some cases with an increased neutrophil-to-lymphocyte ratio and increase in eosinophils[77,78]. In our study 8/12 studies showed a significant increase in WBC in COVID-19 patients with liver injury *vs* those without liver injury. Notably, all evaluated studies with > 100 patients (n = 5) in the liver injury group had a significant

Table 4 Other retrospective cohort studies

Ref.	Country	Setting	Evaluated parameters	Comparison	Summary of main findings	NOS
Cao *et al*[72]	China	Hospitalized patients	WBC, ANC, ALC, DDIM	Elevated ferritin *vs* disease controls	Elevated ferritin corelated with disease severity and degree of liver injury	7
Chen *et al*[73]	China	Hospitalized patients	ANC, ALC, PLT, DDIM	Recurrent cases *vs* disease controls	Elevated AST and ALT are predictive of COVID-19 recurrence	7
Ding *et al*[74]	China	Hospitalized patients	ALC, PLT, DDIM	Deceased *vs* discharged	Abnormal AST and D-Bil were independent predictors of mortality	7
Lei *et al*[75]	China	Hospitalized patients	ANC, ALC, PLT, DDIM	Comparison of parameters for peak levels of each LFT	Associated ANC, ALC, PLT, and DDIM with peak levels of AST, ALC, ALP, and TBIL	6
Luan *et al*[76]	China	ICU hospitalized	WBC, HGB, ANC, ALC, PLTS, DDIM, PT, PTT, INR	CAC *vs* non-CAC	CAC correlates with liver injury	8

ALC: Absolute lymphocyte count; ALP: Alkaline phosphatase; ANC: Absolute neutrophil count; CAC: COVID-associated coagulopathy; COVID-19: Coronavirus disease 2019; D-Bil: Direct bilirubin; DDIM: D-dimer; HGB: Hemoglobin; ICU: Intensive care unit; INR: International normalized ratio; LFT: Liver function test; NOS: Newcastle-Ottawa Scale; PLT: Platelet count; PT: Prothrombin time; PTT: Partial thromboplastin time; WBC: White blood cell count.

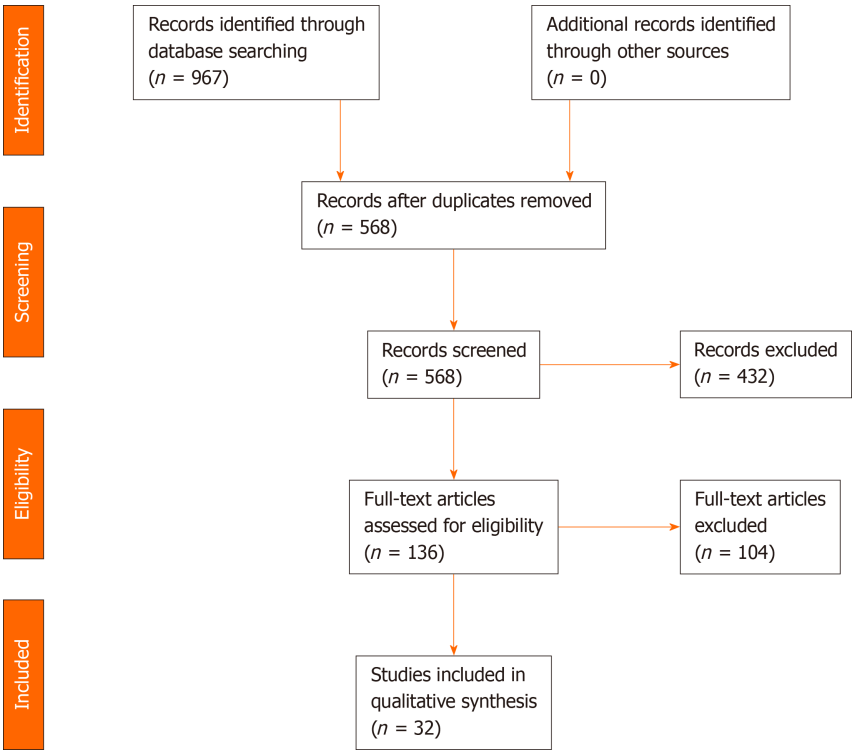


Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2009 flow diagram.

difference in WBC ($P < 0.001$), suggesting that the WBC is a useful addition to LFT for assessing liver function in COVID-19. However, the available data do not appear to support a role for WBC in risk stratification, as only 1/5 studies of non-severe *vs* severe disease demonstrated a significant difference in the WBC.

Lymphopenia, believed to be due to a defective host response, has been identified in COVID-19 patients and has been associated with increased risk of severe disease[2-4,79]. The overall performance of ALC as a biomarker of liver injury appears mixed, as it is not significantly different from COVID-19 patients without liver injury in 4/10 studies with > 100 liver injury patients. Likewise, 3/5 studies showed the ALC to be associated with severe disease and increased risk of liver injury.

Neutrophilia appears to be associated with cytokine storm and hyperinflammation, which has been implicated as a major factor in the pathophysiology of COVID-19. Accordingly, differences in the ANC have been noted in studies evaluating the roles of hematologic data in more generalized populations of COVID-19 patients[3,6,8,9,50,79]. In our study, altered ANC appears to be associated with liver injury in COVID-19, with 5/6 studies with > 100 participants with liver injury having significant differences in ANC compared to those without liver injury. Its usefulness for risk stratification is more difficult to interpret. Although only 1/4 non-severe *vs* severe COVID-19 studies demonstrated a difference in ANC, it has been associated with increased risk of COVID-associated coagulopathy, increased serum ferritin, and increased liver enzymes[72,75,76]. Thus, although ANC appears to be a useful maker of liver injury, its potential as a marker for disease stratification is unclear at this point.

Thrombocytopenia has been identified as a maker of severe COVID-19 and has been associated with consumptive coagulopathy[5,79]. In our study, the majority of studies of COVID-19 with liver injury *vs* those without liver injury did not identify significant difference in PLTs, with only 2/7 studies with > 100 patients in the liver injury group demonstrating significant differences in PLTS. Likewise, 0/4 studies of severe *vs* non-severe COVID-19 patients in our analysis demonstrated significant differences in PLTs. These results indicate that PLTs are not useful either for diagnosis or risk stratification in this group.

An association of decreased HGB with severe COVID-19 has been proposed, but is controversial due to the high heterogeneity of available data[80]. Interestingly, our data imply a potential role of HGB for assessment of liver function in COVID-19, with significant differences in hemoglobin detected in 5/5 studies of liver injury with > 100 participants. Unfortunately, HGB was analyzed in only 1 study in the severe *vs* non-severe study group[70], and a significant difference was not identified. Although these results are promising for the use of HGB as an adjunct to detection of liver injury, further work is necessary to determine whether HGB is a predictor of high risk in this population.

Acquired coagulopathies are a major feature of COVID-19, and a subset of patients develop disseminated intravascular coagulation and multiorgan failure[79,81,82]. In patients with liver injury, significant differences in DDIM were identified in 3/6 studies with > 100 participants; prolongation of PT was identified in 2/4 studies; and prolonged aPTT was present in 0/1 of these studies. In studies comparing non-severe to severe COVID-19 disease differences in DDIM level were identified in 3/3 studies and PT prolongation was identified in 1/1 study. These findings, although based on only a limited number of studies, imply that coagulopathy in COVID-19 is not limited to patients with liver injury and is associated with more severe disease.

Limitations

There were some limitations to this study. Despite an effort to comprehensively search the peer-reviewed medical literature, publications that did not mention liver injury in the title, abstract, or keywords of the papers may not have been included in the search results. Delays in indexing in the databases may also have contributed to papers not being identified in the search. Although some national and international organizations have worked to precisely define the term COVID-19 liver injury[83,84], the lack of precision, especially in papers published early in the pandemic, makes it challenging to confidently compare the results of individual studies.

CONCLUSION

We conducted a systematic review of the peer-reviewed literature regarding laboratory hematology data and their potential use in diagnosis and risk stratification in COVID-19 patients with liver injury. Based on our interpretation of the evidence, evaluation, WBC, ANC, ALC, and HGB may be of interest in addition to LFT in the distinction of patients with liver injury from those without. Since these markers are cited as markers of severe disease in many studies in the medical literature[1], this implies that COVID-19 patients are at increased risk of severe disease. This claim is supported by our evaluation of the medical literature that discusses liver injury in the context of non-severe *vs* severe disease. Our findings support the conclusion that WBC, ANC, ALC, and HGB may be useful for diagnosis and risk stratification in the subset of COVID-19 patients with liver failure, as they are for the general population of patients infected by SARS-CoV-2. Other hematology parameters, such as PLTs, which are widely used in critical care medicine and are a component of the SOFA

score, appear to be less relevant in liver failure patients with COVID-19. Our evaluation of the literature concludes that, although it has a high methodological quality, it is limited by the lack of a clear definition for COVID-19 liver injury, and by the lack of comprehensive reporting of laboratory data in many epidemiological studies. The limited statistical power of a high fraction of these studies, and the lack of statistical analysis of hematologic parameters beyond the univariate t-test are further issues. Further work with larger numbers of patients and more robust data analysis may be of interest to determine the precise role of hematologic parameters in the diagnosis and stratification of COVID-19 patients with liver injury.

ARTICLE HIGHLIGHTS

Research background

Hematology laboratory testing has an established role in risk stratification in patients with coronavirus disease 2019 (COVID-19) disease. Liver injury is common in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The utility of hematology laboratory data in diagnosis and risk stratification of patients with COVID-19-related liver injury is unclear.

Research motivation

To what degree are the abnormalities in hematologic parameters seen in the general population of patients with COVID-19 also seen in the subpopulation of patients with associated liver injury? Is liver injury in COVID-19 indicative of severe disease. Does liver injury correlate with hematologic markers of severe disease? What is the quality of literature that addresses these questions?

Research objectives

The objectives of this study were: To determine the extent to which previously reported hematologic abnormalities are seen in patients with COVID-19-associated liver injury; to assess whether liver injury in COVID-19 is a sign of severe disease, and the extent to which liver injury correlates with markers of severe disease; and based on the extant literature, to determine the quality of this evidence.

Research methods

This study was conducted as a Preferred Reporting Items for Systematic Reviews and Meta-Analyses-compliant systematic review. We extracted information from cohort studies related to the topic of liver injury in COVID-19 in which laboratory hematology data were included.

Research results

In all, 32 articles were included in the systematic review, which consisted of 22 articles with a cohort of COVID-19 patients with liver injury; 5 comparing non-severe *vs* severe COVID-19 populations in which liver injury was addressed and 5 other cohort studies with a focus on liver injury. White blood cell count, absolute neutrophil count, absolute lymphocyte count (ALC), and hemoglobin were the parameters most useful to distinguish COVID-19 with liver injury from COVID-19 without liver injury. ALC and d-dimer were potentially useful in distinguishing non-severe from severe COVID-19. Liver injury was more frequently seen in cohorts with severe disease. Most studies were of high quality (24/48, 86%) with 4/28 (14%) of moderate quality and 0 of low quality.

Research conclusions

The use of select hematologic parameters in diagnosis and risk stratification of liver injury in COVID-19 patients appears warranted. The relevant literature is high quality, but is limited by the small number of studies with high statistical power and the variable definition of COVID-19 liver injury in the literature.

Research perspectives

Future studies, preferably with a prospective design, large numbers of patients, and rigorous definition of liver injury would be useful to validate these findings.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 April 28; 9(2): 208-219

DOI: 10.13105/wjma.v9.i2.208

ISSN 2308-3840 (online)

META-ANALYSIS

Helicobacter pylori infection and susceptibility to cardiac syndrome X: A systematic review and meta-analysis

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Conflict-of-interest statement: No potential conflicts of interest.

PRISMA 2009 Checklist statement: The manuscript was revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Cardiac syndrome X (CSX) is characterized by persistent angina with normal coronary arteries. Several pathophysiologic mechanisms have been introduced, particularly *Helicobacter pylori (H. pylori)* infection.

AIM

To investigate the association between *H. pylori* infection and CSX.

METHODS

All studies related to *H. pylori* infection and CSX were evaluated by comprehensive searches of global databases such as ISI Web of Knowledge, PubMed, Scopus, EMBASE, and Google scholar. Statistical analyses of selected articles were evaluated based on the summary odds ratio (OR). Finally, heterogeneity and publication bias were estimated using the I^2 statistic and Cochrane *Q*-test as well as Begg's and Egger's tests.

RESULTS

A total of 11 studies met our inclusion criteria and 1435 patients (63% female, and 37% male) were reviewed. A significant association was observed between female patients and this syndrome (P = 0.02). Our results showed a positive association between infection with this pathogen and presence of CSX (OR: 5.65; 95% confidence interval [CI]: 4.17-7.64; I^2: 82.20). However, no significant association

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Manuscript source: Invited manuscript

Specialty type: Infectious diseases

Country/Territory of origin: Iran

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: December 30, 2020
Peer-review started: December 30, 2020
First decision: February 28, 2021
Revised: March 3, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: April 28, 2021

P-Reviewer: Koller T, Modun D
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Li X

was observed with *cagA*-positive *H. pylori* strains and this syndrome (OR: 0.97; 0.56-1.70 with 95%CI). Given the heterogeneity and publication bias, the results need to confirmed by further prospective investigation.

CONCLUSION
Based on our results, *H. pylori* infection is associated with an increased risk of CSX. This bacterium appears to play a major role in the pathogenesis of CXS by inducing persistent inflammation.

Key Words: *Helicobacter pylori*; Cardiac syndrome X; Cardiovascular disease; Infection; Meta-analysis; Coronary diseases

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Core Tip: According to various studies, persistent colonization and chronic inflammation caused by *Helicobacter pylori (H. pylori)* infection can lead to systemic disorders outside the gastrointestinal tract. Recently, the role of *H. pylori* in cardiac syndrome X (CSX) has garnered attention. Nevertheless, the mechanism of this syndrome is not fully understood, and it remains controversial. Data from 11 studies were evaluated to assess the association between *H. pylori* infection and CSX. The results showed that there is a positive association between infection with this bacterium and the development of CSX. It seems that *H. pylori* increase the risk of CSX through dysregulation of the immune response and alternating epigenetic events. Statistical analyses showed that the presence of CSX was not dependent on *cagA* status. Several limitations such as small sample size, heterogeneity, and publication bias indicate that confirmation of the present results by further prospective investigation is necessary.

Citation: Karbalaei M, Sahebkar A, Keikha M. *Helicobacter pylori* infection and susceptibility to cardiac syndrome X: A systematic review and meta-analysis. *World J Meta-Anal* 2021; 9(2): 208-219
URL: https://www.wjgnet.com/2308-3840/full/v9/i2/208.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i2.208

INTRODUCTION

Helicobacter pylori (H. pylori) is one of the most important human pathogens in gastric conditions, causing infections in 4-4.5 billion people worldwide[1,2]. This bacterium successfully colonizes in the stomach in childhood and causes chronic infections[3]. After being diagnosed by two Australian physicians, Barry Marshall and Robin Warren in 1994, *H. pylori* was classified as a class I carcinogen[4] by the International Agency for Research on Cancer. Although 85%-90% of infected individuals remain asymptomatic, 10%-15% of cases progress to severe clinical outcomes such as peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma[5]. Most frequently, this bacterium is transmitted through gastro-oral, fecal-oral, or oral-oral pathways[6]. Today, there is ample evidence that the bacteria are isolated from out-of-stomach sites such as atherosclerotic plaques, synovial fluid and urine, suggesting that *H. pylori* plays an important role in extra-intestinal disorders by dysregulating immune response or direct attack[7-14]. According to the literature, *H. pylori* infection is significantly associated with hematological and cardiovascular disorders including iron deficiency anemia, primary immune thrombocytopenia, vitamin B12 deficiency, autoimmune neutropenia, anti-phospholipid syndrome, plasma cell dyscrasias, atherosclerosis, and ischemic stroke[15-22]. Surprisingly, many patients with various disorders such as chest pain, abnormal rest electrocardiogram and positive stress test have normal result of coronary angiogram; this disorder was first named cardiac syndrome X (CSX) by Harvey Kemp in 1973, and then was subsequently termed "angina with normal coronary arteries"[23]. CSX is characterized by a variety of features such as angina-like chest pain, positive exercise testing, positive thallium testing, and normal angiography, and accounts for about 20% of chest pain cases[24-26]. The prevalence of

this syndrome is higher in women than in men[27]. Several pathophysiologic mechanisms have been introduced for CSX including inflammation, endothelial dysfunction, genome polymorphism, dyslipidemia, hypertension, smoking, obesity, vasodilator stimuli, and insulin resistance[28-30]. This syndrome has a poor prognosis and may lead to myocardial infarction following repeated hospitalization[31,32]. The exact mechanism of CSX is not fully understood; however, factors such as chronic inflammation, endothelial dysfunction, and microvascular ischemia appear to be the most important risk factors for progression to CSX[33-35]. On the other hand, the role of infectious agents (both viral and bacterial) in susceptibility to cardiovascular diseases (CVDs) has been suggested[36]. According to extant evidence, *H. pylori* infection leads to CVDs through chronic inflammation and endothelial dysfunction (*i.e.* morphological and functional alterations in carotid artery or atrial stiffness)[37,38]. According to a case report, eliminating this infection could reduce the incidence of CSX[39]. Although several studies have confirmed the link between infection with *H. pylori* and this syndrome, there are some studies with controversial results[40,41]. Because no comprehensive study has been performed on the relationship between this infection and CSX, the present meta-analysis was undertaken to evaluate CSX susceptibility in patients with *H. pylori*.

MATERIALS AND METHODS

Search strategy and inclusion criteria

Initially, a systematic search was performed to evaluate the effects of *H. pylori* infection on susceptibility to CSX. For this purpose, we focused on global databases such as ISI Web of Knowledge, PubMed, Scopus, EMBASE, and Google Scholar, as well as MeSH-based keywords such as "*Helicobacter pylori*," "*H. pylori*," and "Cardiac Syndrome X." Relevant studies were retrieved regardless of publication date and language. The literature search process was conducted separately by two authors (Karbalaei M and Keikha M), and to avoid losing information, the references of each article were evaluated.

Study selection

The inclusion criteria were: cross-sectional, case-control, original, and cohort studies; studies associated with *H. pylori* infection and CSX; studies based on standard diagnostic methods for *H. pylori* infection; and studies on the human population. The exclusion criteria were: review articles, case-reports, and congress abstracts; duplicate studies; repetitive samples; *in vitro* or animal experiments; and unclear or insufficient data. Finally, 11 eligible studies were selected for analysis in this study[41-51].

Quality assessment and data extraction

At this stage, the Newcastle-Ottawa Scale was used to assess the quality of eligible articles. Required information such as first author, publication year, location of study, number of case-control samples, age, gender distribution, infection frequency, diagnostic methods, and reference number are listed in Table 1.

Statistical analyses

We pooled the data using statistical analysis software, Comprehensive Meta-Analysis version 2.2 (Biostat, Englewood, NJ, United States). The association between *H. pylori* infection and incidence of CSX was estimated based on the summary odds ratios (OR) and 95% confidence intervals (Cis). We used the I^2 index and the Cochrane *Q*-test to determine the heterogeneity between studies. Hence, $I^2 > 50$ and Cochrane *Q*-test *P* value < 0.05 were considered significant heterogeneity. Furthermore, in both high and low heterogeneity cases, the random-effects model and the fixed-effects model were used, respectively. Publication bias was also examined through funnel plots and Egger's and Begg's tests.

RESULTS

Literature search and data extraction

In this study, following an initial search in the global databases, we received 206 articles. After evaluating their titles and abstracts and removing duplicate full-text versions, 42 eligible articles were evaluated according to our inclusion criteria, and

Table 1 Characteristics of included studies

Ref.	Publication year	Location	Number of cases		Age		Gender (F/M)		*Helicobacter pylori* infection		Diagnostic method	NOS score
			Case	Control	Case	Control	Case	Control	Case	Control		
Lanza *et al*[41]	2004	Italy	55	60	57 ± 8	57 ± 1	27/28	24/36	53%	64%	ELISA	9
Eskandarian *et al*[42]	2005	Iran	23	23	42 ± 4	41 ± 4	17/6	16/7	91%	48%	UBT	7
Eskandarian *et al*[43]	2006	Iran	40	40	45 ± 5	44 ± 4	29/11	28/12	95%	47.5%	UBT	7
Song *et al*[44]	2008	China	50	50	NA	NA	NA	NA	85.2%	46.7%	UBT	4
Said MohammadZade *et al*[45]	2009	Iran	54	168	54.5	55.2	42/12	125/43	81.48%	56.54%	ELISA	7
Assadi *et al*[46]	2009	Iran	30	30	53.2	52.6	18/12	18/12	50%	0%	UBT	6
Rasmi *et al*[47]	2012	Iran	88	97	53.8	45.7	56/32	61/36	98.86%	57.73%	ELISA	9
Rasmi *et al*[48]	2012	Iran	100	100	51.8	48.9	61/39	61/39	92%	56%	ELISA	9
Rasmi *et al*[49]	2013	Iran	60	60	51.8	48.9	38/22	39/21	95%	40%	ELISA	7
Rasmi *et al*[50]	2016	Iran	88	97	53.8	45.7	56/32	61/36	93.18%	57.73%	ELISA	6
Yu *et al*[51]	2018	China	61	61	NA	NA	NA	NA	92.8%	57.6%	ELISA	5

NA: Not available.

finally 11 articles were selected (Figure 1).

All selected studies were conducted during 2004-2018, and the geographical distribution of the articles was limited to some countries such as Iran, China, and Italy. In eligible studies, the enzyme-linked immunoassay and urea breath test were used to diagnose *H. pylori*. In this meta-analysis, data from 1,435 participants including 649 CSX patients and 786 controls were evaluated. All cases of CSX were diagnosed by typical history of angina with normal or near normal coronary angiography plus a positive none-invasive test or abnormal ECG diagram. Of all patients, 36.61% were male and the rest were female. There was a significant relationship between female sex and getting CSX (P = 0.02); the mean ages of patients and the controls were 51.48 and 48.81, respectively. Despite the positive correlation in most studies, in three studies, this relationship was inconsistent, even the relationship between the *cagA* gene and CSX[47-49]. In general, all eligible studies evaluated factors including hypertension, diabetes, hyperlipidemia, smoking, systolic/diastolic blood pressure values, serum homocysteine levels, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride.

Association between H. pylori and CSX

Statistical analyses showed that the frequency of infection with *H. pylori* in CSX patients was 77.3% (95%CI: 71.7-82, P = 0.01, I^2 = 89.47, Q = 66.51, P = 0.01; Begg's P = 0.86; Egger's P = 0.01), while that of infection in healthy individuals was 54.2% (95%CI: 50-58.3, P = 0.05; I^2 = 60.7, Q = 17.82, P = 0.01; Begg's P = 0.13; Egger's P = 0.03). Based on the summary OR, we found a strong positive association between this infection and CSX (OR: 5.65, 95%CI: 4.17-7.64, P = 0.01, I^2 = 82.20, Q = 56.19, P = 0.01; Begg's P = 0.40; Egger's P = 0.21), suggesting that patients infected with *H. pylori* might be more susceptible to progression to CSX (Figure 2).

CagA is regarded as one of the main bacterial factors causing alterations in normal signaling pathways and dysregulation of actin-rearrangement, as well as induction of chronic and severe immune responses[52,53]. Based on statistical analyses, no significant relationship was observed between *cagA*-positive strains and susceptibility to CSX (OR: 0.97; 95%CI: 0.56-1.70; P = 0.93, I^2 = 0.00, Q = 0.006, P = 0.96). According to the literature, hypertension and hyperlipidemia are the most important CSX-related factors caused by chronic inflammation[31,54,55]. *H. pylori* infection *via* dysregulating the immune system (MALT lymphogenesis) may lead to disruption of normal homeostasis and basal metabolism, and eventually cardiovascular risk[56-60]. Our

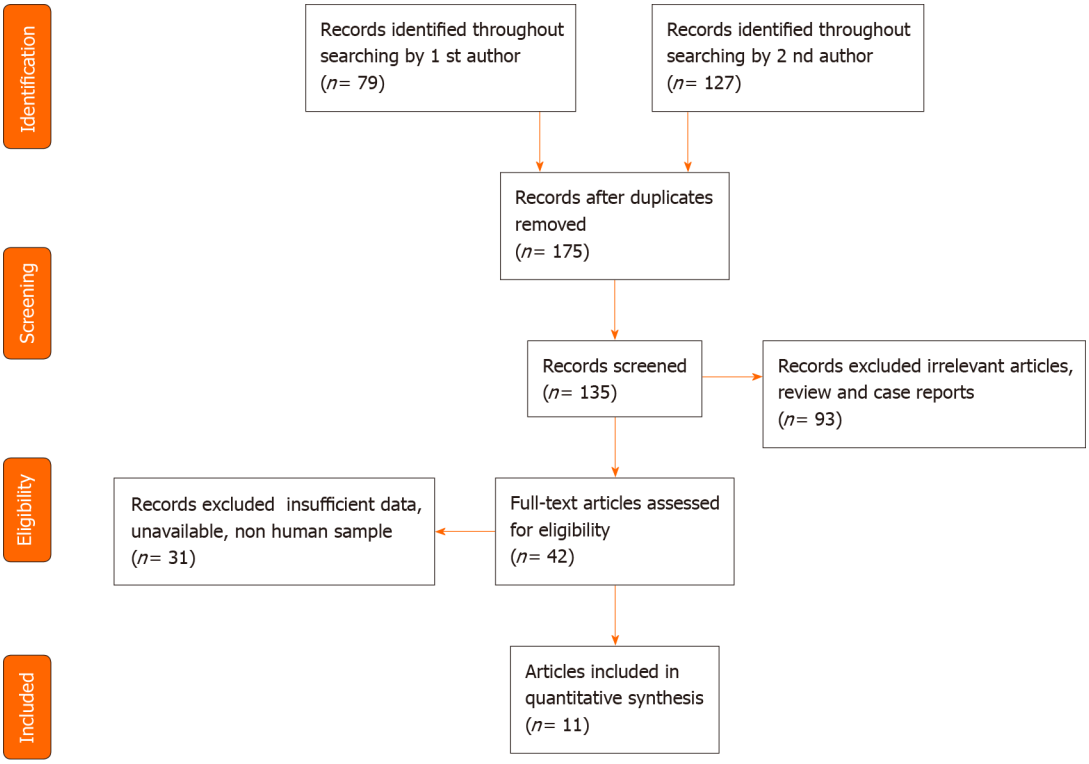


Figure 1 Flow-diagram of literature search and study selection.

Study name	Statistics for each study				
	Odds ratio	Lower limit	Upper limit	*Z* value	*P* value
Lanza et al	0.646	0.306	1.361	-1.150	0.250
Eskandarian et al (1)	11.455	2.167	60.559	2.870	0.004
Eskandarian et al (2)	21.000	4.451	99.082	3.846	0.000
Lunan et al	6.390	3.254	12.549	5.386	0.000
Mohammadzadeh et al	3.381	1.595	7.167	3.178	0.001
Assadi et al	61.000	3.418	1088.585	2.796	0.005
Rasmi et al (1)	63.696	8.518	476.310	4.047	0.000
Rasmi et al (2)	9.036	3.966	20.584	5.240	0.000
Rasmi et al (3)	28.500	7.998	101.554	5.167	0.000
Rasmi et al (4)	10.006	3.981	25.149	4.898	0.000
Zhengfeng et al	10.586	3.407	32.891	4.079	0.000
Meta analysis	5.650	4.177	7.643	11.236	0.000

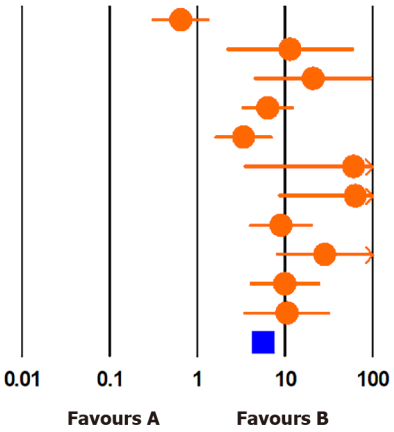


Figure 2 The forest plot represents a probable association between *Helicobacter pylori* infection and susceptibility to cardiac syndrome X.

results also indicate a positive significant relationship between infection caused by this bacterium and the presence of hyperlipidemia/ hypertension in CSX patients (OR: 3.33; 95%CI: 1.92-5.76; and OR: 2.30; 95%CI: 1.37-3.87, respectively). About 14.1% of CSX patients were smokers; however, there was no significant association between smoking and the risk of CSX (OR: 0.95, 95%CI: 0.62-1.45, P = 0.83).

Publication bias

In the present meta-analysis, we evaluated publication bias using several tests such as Begg's, Egger's, and funnel plot evaluation (Figure 3). According to the results, there was significant publication bias that seems to be due to the low number of available articles.

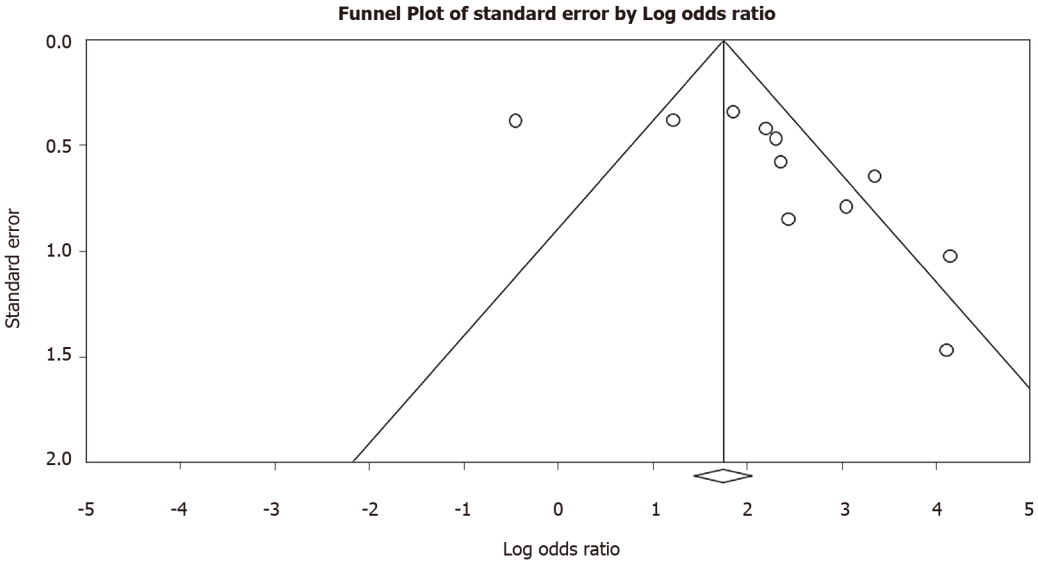


Figure 3 Funnel plot of the meta-analysis on the association between *Helicobacter pylori* infection and cardiac syndrome X.

DISCUSSION

CSX is one of the heterogeneous syndromes in which cardiac (malfunction of Na-H+ pump, smooth muscle cell migration, endothelial dysfunction, *etc.*) and non-cardiac (sympathetic reactions, inflammation) mechanisms are involved[61]. Although CSX is a multifactorial disease, the most important pathological factors associated with this syndrome are endothelial dysfunction, coronary artery disease, and myocardial ischemia[62,63]. Recently, the role of inflammation in pathogenesis of CSX, has been defined[64,65]. Pro-inflammatory cytokines, especially interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), cause several pathological changes, such as endothelial dysfunction, motility vessels, and microvascular stenosis, which in turn predispose to CSX[66]. In general, infectious agents are considered risk factors for CVDs due to long-term stimulation of the immune response[67]. Today, the role of *H. pylori* infection in CVDs immunopathogenesis has received increasing attention[68]. Immune responses (*e.g.*, cytokines and reactive oxygen species) against this pathogen lead to extracellular disorders[69,70]. Innocenti *et al*[71] showed that this bacterium can activate endothelial cells. In another study, Oshima *et al*[72] found that complications such as systemic inflammation and endothelial dysfunction were more common in patients infected with *H. pylori* than in healthy individuals. In this study, we performed a comprehensive analysis of this association, and finally the results showed that there is a meaningful positive relationship between infection with this bacterium and CSX (OR: 5.65; 4.17-7.64 with 95%CIs). In their study, Rasmi *et al*[47] also showed a significant correlation between these two events in the Iranian population. Moreover, according to a study by Nocente *et al*[39], eliminating infection can significantly contribute to CSX regression. Our results also showed that the rate of *H. pylori* infection is higher in CSX patients than in nested-healthy controls (77.3% *vs* 54.2%). The bacterium contains various virulence factors that act as bacterial super-antigens, and stimulate lamina propria macrophages and local T cells[73]. During the infection, inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α increase the infiltration of leucocytes, and aggregation of platelets and PMNs into the site of infection. Bacteria cause some events such as platelet activation and endothelial dysfunction by increasing von Willebrand factor[74,75]. Chemokine receptors such as E-selectin, P-selectin, and intercellular adhesion molecule 1 damage endothelial vessels by increasing the influx of leukocytes[76]. Cannon *et al*[77] showed that the number of chemokine receptors in CSX patients was higher than that in the control group. Other parameters such as serum fibrinogen level, and white blood cell or platelet count in patients with long-term infection are significantly higher than those in the control group[78,79]. Lanza *et al*[41] found that IL-1/C-reactive protein levels were higher in CSX patients than in the healthy group, so they identified these as CSX biomarkers. Chronic infection may cause several disorders, such as motility vessel, contraction of small vessels, and eventually endothelial dysfunction, by inducing inflammation and increasing immune responses[80-82]. The phenomenon of cross-reaction between *H. pylori* antigens and autoantigens increases endothelial damage[83]. Although CagA

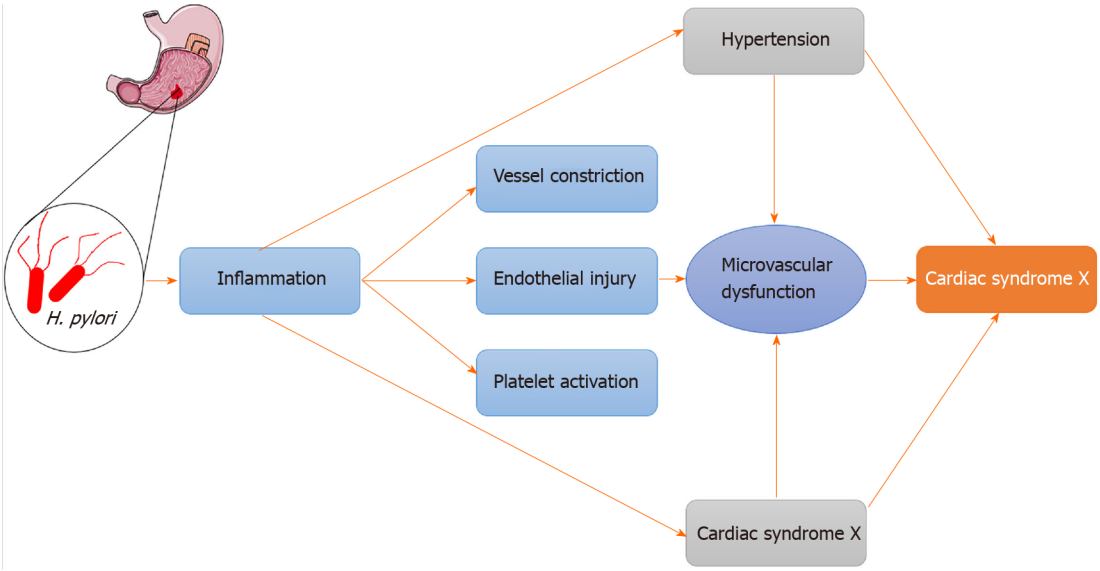


Figure 4 Main involved mechanisms of *Helicobacter pylori* infection in susceptibility and progression to cardiac syndrome X.

antigen is a known virulence factor that is associated with complications such as peptic ulcer disease and gastric cancer, no significant relationship was found between the *cagA* status and CSX disease (OR: 0.97; 0.56-1.70 with 95%CIs). Risk factors such as hypertension, hyperlipidemia, diabetes mellitus, smoking, and alcoholism are among major risk factors for CSX[84,85]. In general, inflammatory responses to chronic infections dysregulate the basal metabolism; for example, IL-6 affects the liver and increases gluconeogenesis and synthesis of fatty acids[86-90]. Another inflammatory cytokine, TNF-α, reduces the clearance of blood fat particles by blocking liver lipoprotein lipase, thus, it is associated with hyperlipidemia[91-93]. Nowadays, there is ample evidence of a positive correlation between chronic *H. pylori* infection and hypertension. In the study conducted by Migneco *et al*[94], it was shown that complete eradication of infection with these bacteria significantly reduced hypertension in infected patients[93]. Based on previous studies, this bacterium affects the immune system, leading to defects in lipid metabolism and eventually hyperlipidemia[95-98]. In general, prolonged *H. pylori* infection leads to extra-gastrointestinal complications such as motility vessel, microvascular constriction, endothelial dysfunction, hypertension, and hyperlipidemia, and at the end CSX (Figure 4).

Limitation

Our meta-analysis had several limitations such as: small number of participants, limited geographical distribution, high heterogeneity, significant publication bias, and lack of adjustment for covariates such as age and CV risk factors, nature of studies (case-control and no cohort), and lack of access to raw data such as socio-economic status, alcohol consumption, and environmental hygiene. However, due to heterogeneity, we need further research to confirm the present findings.

CONCLUSION

The present study was the first comprehensive study to evaluate the relationship between *H. pylori* infection and CSX. Overall, our results suggested an association between *H. pylori* infection and the risk of CSX; however, we need further research with larger sample sizes to confirm the present findings. In this study, using a systematic review, we showed that *H. pylori* infection increases the risk of developing the CSX disease through inflammation, endothelial damage, and the effect on related risk factors.

ARTICLE HIGHLIGHTS

Research background

Cardiac syndrome X (CSX) is multilateral condition that not well characterized.

Research motivation

For the first time, we investigated the probable association between *Helicobacter pylori (H. pylori)* infection and risk of develop to cardiac syndrome.

Research objectives

In the present study, the association between *H. pylori* infection and CSX was investigated.

Research methods

We searched several databases to collecting all available documents relevant to impact of *H. pylori* infection and susceptibility to CSX. odds ratio (OR) is used for estimation of probable link between *H. pylori* infection and CSX.

Research results

There is a positive association between *H. pylori* infection and risk of develop to CSX (OR: 5.65, 95%CI: 4.17-7.64, I^2: 82.20).

Research conclusions

In this study, we found that *H. pylori* infection increases the risk of CSX. We showed that there is no significant relationship between *cagA* status and progression to CSX. The frequency of vascular endothelial destructive risk factors such as hyperlipidemia and hypertension in patients infected with *H. pylori* was significantly higher than the control group.

Research perspectives

Infectious agents such as *H. pylori* infection can provide a condition to susceptibility of CSX.

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ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 June 28; 9(3): 220-326



Published by Baishideng Publishing Group Inc

World Journal of Meta-Analysis

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The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

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PUBLICATION DATE
June 28, 2021

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 June 28; 9(3): 220-233

DOI: 10.13105/wjma.v9.i3.220

ISSN 2308-3840 (online)

REVIEW

Post COVID-19 infection: Long-term effects on liver and kidneys

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Author contributions: Srivastava S and Garg I contributed to literature review, manuscript writing and proof reading.

Conflict-of-interest statement: The authors declare have no conflict of interest.

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Manuscript source: Invited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: India

Peer-review report's scientific quality classification

Grade A (Excellent): 0

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Abstract

Coronavirus disease 2019 is a pandemic, which has affected millions of people across the globe in the year 2020. This disease is caused by a virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that belongs to the family of coronaviruses and primarily affects the respiratory system. This infection has a wide spectrum of clinical manifestations ranging from asymptomatic form to mild, moderate and severe forms depending upon the age, comorbidity and immunity of an affected individual. Hyper-inflammatory response due to SARS-CoV-2 adversely affect several internal organs. Besides lung injury, which is the main outcome of SARS-CoV-2 infection, it has been reported to adversely impact other organs including the liver and kidneys. SARS-CoV-2 virus can also have a direct adverse impact on liver as well as kidneys due to systemic inflammatory response or drug toxicity, leading to elevated levels of liver injury markers and acute kidney injury. Clinical outcomes of SARS-CoV-2 infection could be worse in patients suffering from pre-existing liver and kidney disease. So far, there have been several reports on the mechanism of liver and kidney injury during SARS-CoV-2 viral attack. However, the long-term impact of this infection on these organs is yet to be understood. This review summarizes the possible causes and effects of SARS-CoV-2 on the liver and kidneys during the infection and post recovery based on available literature.

Key Words: Alanine aminotransferase; Aspartate aminotransferase; Acute Kidney injury; COVID-19, Kidneys; Liver; SARS-CoV-2

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Core Tip: Coronavirus disease 2019 (COVID-19) infected patients with pre-existing liver and kidney comorbidities are likely to have a poorer clinical prognosis and are at higher risk of severe infection and increased mortality. Data indicates that COVID-19 infection causes acute kidney and liver damage. However, its long-term consequences

Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): E

Received: January 25, 2021
Peer-review started: January 25, 2021
First decision: April 19, 2021
Revised: May 7, 2021
Accepted: June 4, 2021
Article in press: June 4, 2021
Published online: June 28, 2021

P-Reviewer: Gracia-Ramos AE, Khader MA
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Li JH



are yet to be elucidated. Currently in the absence of specific therapy for this viral infection, further clinical studies are needed to understand COVID-19 pathology associated with liver and kidneys. The present review summarizes the effects of COVID-19 on the liver and kidneys during infection and post recovery.

Citation: Srivastava S, Garg I. Post COVID-19 infection: Long-term effects on liver and kidneys. *World J Meta-Anal* 2021; 9(3): 220-233
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/220.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.220

INTRODUCTION

Coronaviruses are a large family of viruses causing a variety of diseases. In recent times, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome-CoV) have caused respiratory illness (pneumonia) to thousands of people worldwide, leading to death of many patients. Novel coronavirus-induced pneumonia caused due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was declared a Public Health Emergency of International Concern on January 30, 2020[1] and later named as coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO) on the February 11, 2020. SARS-CoV-2 (COVID-19) viral infection was first reported in December 2019 in Wuhan, Hubei Province, China (CDC 2019), and it spread to almost all the parts of the world in a short span of time.

COVID-19 infection causes severe pulmonary disease and has devastating effects. Itinduces a systemic disease attacking multiple organs, potentially causing major damage, including mortality and long-term effects. The mortality rate due to COVID-19 infection is 2%-5% in the general population. However, patients with comorbidities, such as hypertension, diabetes and chronic obstructive pulmonary disease, are considered at higherrisk, with a mortality rate above 50%[2].

There is no definite treatment for infection caused by SARS-CoV-2. Therapeutics currently under consideration by the WHO includeinterleukin6-blockers, colchicines, monoclonal antibodies, anticoagulants and vitamin D along with systemic corticosteroids in patients having severe and critical condition[3]. However, treatment protocols across different countries vary wherein several antiviral candidates are in use for the treatment of infected patients like, the Food and Drug Administration approved remdesivir[4], corticosteroids, oseltamivir (Tamiflu), arbidol hydrochloride, hydroxychloroquine and convalescent plasma therapy[5], although the WHO does not recommend these treatment strategies. Several broad-spectrum antibiotics along with anti-inflammatory drugs are given to prevent acute respiratory distress syndrome. There has been a worldwide endeavour to design a safe and effective vaccine for protection against it. In this regard, several potential vaccines are under various stages of clinical trials.

The infection caused by SARS-CoV-2 virus is predominantly a respiratory disease. However, its adverse effects on other organ system remains unclear. The most common clinical presentation includes respiratory tract involvement with mild to high fever, shortness of breath and cough. However, SARS-CoV-2 infection has a wide spectrum of symptoms ranging from asymptomatic cases and very mild cases with minor symptoms of sore throat and loss of smell or taste[6] to acute respiratory failure and damage to other organs including acute kidney injury (AKI), liver damage, cerebrovascular stroke and gastroenteritis[7,8]. There are several reports on adverse effects of SARS-CoV-2 infection on liver and kidney functions causing liver injury and acute renal injury. The purpose of this review is to ascertain the damaging effects of SARS-CoV-2 infection on the liver and kidneys.

ORIGIN, TRANSMISSION AND PATHOGENETIC MECHANISM OF SARS-COV-2

Coronaviruses are enveloped viruses with a positive sense single-stranded RNA genome with sizes between 26-32kb[9]. Four genera of coronavirus viz., α, β, γ, δ have

been identified so far, with human coronaviruses detected in α coronavirus and β coronavirus genera[10]. SARS-CoV-2 is a positive-stranded RNA virusbelonging to the genus Beta-coronavirus. It has a crown like structure due to the presence of spike glycoproteins on the envelope[11]. Scientists are trying to find the animal host of this novel coronavirus. However, most groups agree that the intermediate hosts are bats, pangolins or sea animals[12]. Sequencing of the SARS-CoV-2 genome and its phylogenetic analysis revealed that it has 88%-89% similarity with two bat-derived SARS-like coronaviruses, bat-SL-CoVZC45 and bat-SL-CoVZXC21[12].

The virus SARS-CoV-2 mostly enters the body through inhalation, binds to epithelial cells in the nasal cavity and starts replicating[13]. Like SARS-CoV, angiotensin converting enzyme 2 (ACE2) acts as the main receptor for SARS-CoV-2, which mediates internalization of the virus[14]. The ACE2 protein is expressed in a variety of human cells, including type II alveolar cells, oral, oesophageal, ileal epithelial cells, myocardial cells, proximal tubule cells of the kidneys as well as urothelial cells of the bladder. The cleavage of the spike (S) protein of SARS-CoV-2 by a cellular enzyme named furin at the S1/S2 site is essential for viral entry to the lung cells[15]. The activated S protein is primed by *TMPRSS2* and gets attached to ACE2 receptors. The virus replicates and migrates down the respiratory tract along the conducting airways, thus triggering the innate immune response. In the next stage of infection, the virus further reaches gas exchange units of the lung and infects alveolar type II cells.

CLINICAL MANIFESTATIONS OF SARS-COV-2

Although individuals of any age group can succumb to SARS-CoV-2 infection, susceptibility largely varies according to age and comorbidities. Its common clinical features in adults include fever, dry cough, sore throat, headache, fatigue, myalgia and breathlessness[16,17]. The disease manifestations may range from mild pneumonia to moderate pneumonia, which may result in hypoxia requiring hospitalization and critical illness requiring mechanical ventilation, multiorgan dysfunction and possibly death[18]. Individual age, underlying comorbidities and severity of the disease may increase the risk of death due to SARS-CoV-2 up to 49% in critically ill patients[19]. However, the actual mortality rate due to this infection varies in different parts of the world.

IMPACT OF COVID-19 ON LIVER

Liver is a vital organ in the human body. COVID-19 infection can either have serious impact on individuals with pre-existing liver disease or can have direct adverse effects on the liver (Figure 1). Although several recently published reports establish that patients with liver diseases are at increased risk and severity of COVID-19 infection, interaction of pre-existing liver disease with SARS-CoV-2 has not been investigated in detail. There have been several studies to demonstrate adverse effects of SARS-CoV-2virus on the liver, and its impairment post SARS-CoV-2 infection is also an emerging concern. Previous studies of SARS coronavirus have shown that up to 60% of patients had a liver impairment showing viral nucleic acid and damage in a liver biopsy[20-22]. Authors in these studies noted that this could be due to treatment of patients with high doses of antibiotics, hepatotoxic antiviral drugs and steroids. Because the process of RNA shedding is well described in the gastrointestinal tract and the production of ACE2 is higher in the colon, biliary system and liver, it is very likely that SARS-CoV-2 may have active replication in these sites, which might in turn result in direct or indirect tissue injury. Because liver is one of the potential entry targets for SARS-CoV-2, the liver damage caused due to infection by SARS-CoV-2 can be attributed to several factors including direct damage by penetrating virus, inflammatory or immune response, increased risk of thrombosis and liver lesions caused by anti-COVID-19 drug therapy[15,23].

Several independent reports are available to demonstrate liver function abnormalities in COVID-19 patients. Elevated levels of liver injury markers such as alanine aminotrans-ferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferaseand total bilirubin levelshave been reported in patients of SARS-CoV-2[24-30]. A few selected cohort studies have been summarized in Table 1 showing levels of AST and ALT in COVID-19 patients having liver abnormalities either at the time of viral infection or post viral infection.

Table 1 Studies showing liver function abnormalities in coronavirus disease 2019 patients

No.	Study	Finding	Ref.
1	Large scale study: *n* = 1099	AST/ALT were elevated in 18.2%/19.8% of mild COVID-19 patientsand 39.4%/28.1% in severe COVID-19 patients	Guan *et al*[7]
2	Small scale study: *n* = 41	AST was elevated in 62% of patients in the ICU compared with 25% in those who did not require care in the ICU	Huang *et al*[8]
3	*n* = 99	AST (U/L): All patients: 34 (26-48); ALT (U/L): All patients: 39 (21-55)	Chen *et al*[17]
4	*n* = 113	AST (U/L): All patients: 16 (22-46); ALT (U/L): All patients: 22%; Deaths: 27%	Chen *et al*[25]
5	*n* = 115	AST (U/L): All patients: 28.3 ± 15.6; ULN ≤ 50 U/L: 85%; 50-150 U/L: 15%; > 150: None; ALT (U/L): All patients: 34 (18-67); Moderate: 28 (21-43.5); Severe: 36.5 (17.5-71.5)	Zhang *et al*[30]
6	French Cohort Study, *n* = 281 patients	102 (36.3%) patients had liver dysfunctions. High level of GGT was the most common perturbation (25.3%). Elevated levels of AST (24.3%) and ALT (12.8%)	Chaibi *et al*[81]
7	Large scale study: China, *n* = 350	ALT (U/L) and AST (U/L) were profoundly elevated in critically ill patients (33; 49) in comparison to severe patients (23; 29) and mild patients (22; 26)	Fu *et al*[82]
8	*n* = 79	AST (U/L): All patients: 30 (20-50); Moderate: 28 (22-48); Severe: 35 (22-55). ALT (U/L): All patients: 34 (18-67); Moderate: 28 (21-43.5); Severe: 36.5 (17.5-71.5)	Xie *et al*[83]
9	*n* = 104	ALT (U/L) and AST (U/L) were profoundly elevated in patients (36.5; 33)	Li *et al*[84]
10	Large scale study: China, *n* = 657	ALT (U/L) was highly elevated in critically ill patients (41) in comparison to moderate patients (25)	Wang *et al*[85]
11	Large scale study: China, *n* = 1827	AST (U/L): Abnormal in pre-hospitalization patients (20.3%); Admission (66.9%) and peak hospitalization (83.4%) patients with COVID-19; ALT (U/L): Abnormal in pre-hospitalization patients (19.1%); Admission (41.6%) and peak hospitalization (61.6%) patients with COVID-19 infection	Hundt *et al*[86]
12	Large scale study: New York, *n* = 5700	58.4% developed AST values > 40U/L and 39% ALT > 60U/L. 56 patients (2.1%) developed acute hepatic injury defined as an elevation in AST or ALT of >15 times the upper limit of normal	Richardson *et al*[87]

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019; GGT: Gamma-glutamyl transferase; ICU: Intensive care unit; ULN: Upper limit of normal.

Apart from cohort studies, numerous case reports and meta-analyses are also available, which depict severe clinical outcome of viral infection to those patients who were having pre- and post-liver abnormalities[31,32]. Liver injury in SARS-CoV-2 infection is one side of the coin, but little is known about SARS-CoV-2 clinical presentation in the context of liver transplant. It is a well-known fact that these patients are immunosuppressed and are more susceptible to various opportunistic infections. Few case reports are available, which mainly focused on patients who underwent liver transplant a few months or year before SARS-CoV-2 infection but after getting SARS-CoV-2 infection, disease progressed rapidly from mild to critical stage and ultimately led to death despite giving various therapies and treatment[33-35].Few case reports showed promising recovery of these kinds of patients after dynamic surveillance and treatment[35,36]. There is a prerequisite to plan strategies to manage SARS-CoV-2 infection in the post-transplant situation to reduce mortality.

Youssef *et al*[37] reported a meta-analysis of 3428 patients having SARS-CoV-2 infection from 20 retrospective studies and found that these patients exhibited elevated levels of ALT and AST. They concluded that dysfunctionality of the liver is associated with a critical outcome of SARS-CoV-2 infection, and precise monitoring is very much required in these patients to avoid serious clinical outcomes[37]. Recent meta-analysis done by Del Zompo *et al*[38] included 20724 COVID-19 patients out of 36 studies and showed that there is an elevation of AST 26.5% and ALT 22.8% at the time of admission. In another meta-analysis, it has been also demonstrated that liver enzymes are firmly linked with 12882 confirmed COVID-19 patients as they showed that COVID 19 infected patients has elevated levels of AST (41.1%) and ALT (29.1%)[39]. It has been reported that COVID-19 associated liver injury is more common in severe COVID-19 than non-severe COVID-19.Wong *et al*[40] reported the pooled odds ratio for elevated ALT (odds ratio = 2.5, 95% confidence interval: 1.6-3.7, I^2 = 57%) and AST (odds ratio = 3.4, 95% confidence interval: 2.3-5.0, I^2 = 56) were higher subjects in critical 5961 COVID-19 patients.

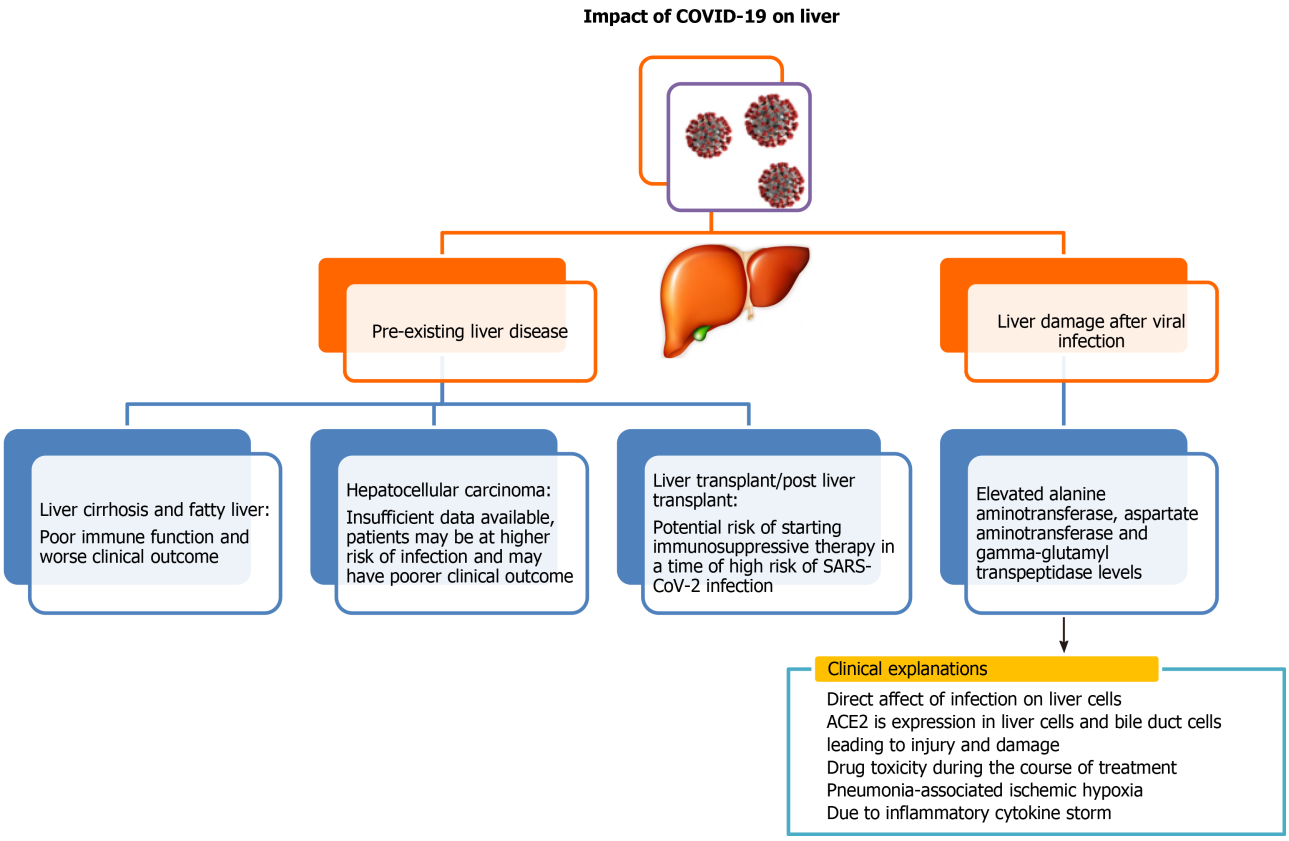


Figure 1 Liver injury due to coronavirus disease 2019 attack. Complications in people with pre-existing liver disease and effects of direct attack of severe acute respiratory syndrome coronavirus 2 virus on liver. ACE2: Angiotensin converting enzyme 2; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Díazet al[41] performed a meta-analysis to characterize hepatic pathological findings in COVID-19 patients. They included 18 studies, which were all case reports and case series from autopsies and reported that 55.1% of patients had hepatic steatosis, 34.7% had congestion of hepatic sinuses, 29.4% had venous thrombosis, 20.5% patients were with fibrosis, 13.5% patients represented Kupffer cell hyperplasia, 13.2% had portal inflammation, and 11.6% had lobular inflammation. Thus, there was a high prevalence of hepatic steatosis and vascular thrombosis as major histological liver features[41].

Finally, there is an utmost need to pay more attention towards the occurrence of liver damage in the diagnosis and treatment of SARS-CoV-2 infection. It is also advisable to clinicians to closely monitor the progression of liver dysfunction in mild to moderate as well as severe COVID-19 patients so that appropriate medical treatment could be given based on severity. It has become of utmost importance to get deep insight into the COVID-19 pathogenesis of liver in patients pre- and post-infection to develop an effective treatment regime for patients.

Liver impairment in alcoholic and nonalcoholic COVID-19 patients

Scanty information is available regarding liver impairment in alcoholic COVID-19 patients in comparison to nonalcoholic COVID-19 patients. Although, it is well known that alcohol is a major hepatotoxin, and its consumption is responsible for more than 40% of all deaths from liver disease as it interferes with the immune system and compromises its function[42]. A wide spectrum of liver disease ranging from mild to severe comes under the umbrella of alcoholic hepatitis, which increases the risk of severe COVID-19 infection. It has been observed that the patients with alcoholic liver disease infected with SARS-CoV-2 have a worse prognosis. As we are evolving everyday for current pandemics, there are not straight and clear recommendations for the management of alcoholic liver disease in the COVID-19 pandemic[43]. There is little information on the interaction of alcoholic hepatitis and COVID-19[44]; however, it is well-known that patients with advanced alcoholic liver disease are more prone to develop numerous kinds of respiratory distress[45].

COVID-19 AND AKI

Coronavirus infections have been associated with multiple organ dysfunction, including AKI (Figure 2). Several reports have shown higher frequencies of renal abnormalities in COVID-19 patients. Some studies have classified kidney impairment as an independent risk factor for mortality in COVID-19 patients admitted to hospitals [46,47]. It is important for clinicians to understand whether accompanying AKI phenomenon occurs due to infection or is an important marker of disease severity[48]. AKI occurrence at an early stage is considered a negative prognostic factor for survival and is more common in severely infected patients, especially those admitted to an intensive care unit (ICU)[49,50]. Individuals with no underlying kidney problems also show signs of kidney damage after getting infected with SARS-CoV-2. Renal injury by SARS-CoV-2 can also be attributed to multiple factors such as direct injury due to virus infection or due to systemic effects including host immune clearance and immune response, endothelium-mediated vasculitis, thrombus formation, glucose and lipid metabolism disorder and hypoxia[51]. In the early stages of pandemic spread, incidence of AKI was reported to be in 3% to 9% of patients. However, later studies reported an incidence rate of 15%[52,53]. The COVID-19 associated AKI is managed by avoiding nephrotoxic drugs and by including supportive treatment like renal replacement therapy[47]. Continuous renal replacement therapy by hemofiltration and hemodiafiltration has been used in the past to treat SARS, Middle East respiratory syndrome and sepsis. Thus, it is speculated that continuous renal replacement therapy may be beneficial in patients with COVID-19[54]; however, it needs to be critically evaluated.

Studies report that normal kidneys and intestinal tract have higher ACE2 expression compared to the lungs[28]. ACE2 receptors on the cell walls of the kidney allow the entry of virus into the cells. Thus, kidneys can be directly impacted by the SARS-CoV-2 virus. Diao *et al*[55] examined post-mortem kidney tissue from 6 infected patients and observed severe acute tubular necrosis and lymphocyte infiltration[56]. Additionally, SARS-CoV-2 nucleocapsid protein and clusters has been detected in kidney tubules by immunohistochemistry[55] and in the tubular epithelium and podocytes using electron microscopy[56]. Thus, this pathogenetic nature of a virus causing injury in tubular, glomerular and vascular parts of the kidney leads to AKI. In a recent observational study of 85 deceased patients, it was found that 54% of the patients had severe AKI, and non-recovery from severe AKI was associated with the presence of pigmented casts, as found on kidney biopsies. Researchers further reported that inflammatory markers and medications were associated with specific histopathologic findings in patients dying due to SARS-CoV-2 virus infection[57]. Some selected studies showing kidney abnormalities in COVID-19 patients have been listed in Table 2.

COVID-19 in chronic kidney diseasepatients

Patients with chronic kidney disease have functional defects in innate and adaptive immunity, thus having a persistent proinflammatory state. These patients are at higher risk of infections in the upper respiratory tract; hence they could be prone to severe SARS-CoV-2 infection[47]. Although, no systematic study has been done on this so far, Henry and Lippi[58] found a significant association of chronic kidney disease with severe COVID-19while analysing different available studies.

COVID-19 effect on chronic dialysis patients

Comorbidity such as nephropathy could lead to poor prognosis of COVID-19. Wang [59] studied 230 haemodialysis (HD) COVID-19 patients at Renmin Hospital, Wuhan University. They found 37 individuals amongst them were infected with SARS-CoV-2. Most of these patients presented mild symptoms and did not required admission to an intensive care unit. During this observational study, 7HD patients died, out of which 6 died due to SARS-CoV-2 infection. Another study on HD patients revealed that the most common symptoms of COVID-19, such as fever, cough, and dyspnoea, were not present in these patients. Infact, diarrhoea was the most common symptom, which made the diagnosis even more difficult[60]. In a large-scale retrospective study of 7154 HD patients, SARS-CoV-2 infection was found in 2% of patients, and only about 50% of them had fever while about 20% were asymptomatic. Also, the mortality rate was as high as 31%, much higher compared to the general population[61]. Studies report that HD patients with SARS-CoV-2 infection have reduced inflammatory response, such as levels of circulating CD4 and CD8 T cells, natural killer cells and proinflammatory cytokines and mild symptoms with a lower risk of acute respiratory distress syndrome

Table 2 Studies showing kidney abnormalities in coronavirus disease 2019 patients

No.	Study	Finding	Ref.
1	*n* = 191 patients; 137 were discharged and 54 died in hospital	The incidence of AKI was in 15% in the COVID-19 infected patients, out of which 50% were non-survivors and 1% were survivors	Zhou *et al*[2]
2	*n* = 41 patients	10% patients had elevated creatinine level (> 133 μmol/L) on admission and 7% had AKI. Incidence rate was further increased to 23% in critically ill patients in ICU	Huang *et al*[8]
3	*n* = 193 patients	After hospital admission, 59% of patients developed proteinuria, 44% with haematuria, 14% with increased levels of blood urea nitrogen, and 10% with increased levels of serum creatinine	Li *et al* [46]
4	*n* = 701 consecutive hospitalized patients	11.9% of those with elevated baseline creatinine developed AKI compared to 4.0% in patients with normal baseline creatinine. 44% of patients had proteinuria and haematuria. Mortality rate was higher in patients with proteinuria, haematuria, elevated baseline creatinine and urea, and AKI stage 2-3	Cheng *et al*[47]
5	Observational study of *n* = 287 patients	Most patients recover from AKI stage 1. Patients who progress to AKI stage 2 or 3 have a very high mortality rate	Xiao *et al* [53]
6	Retrospective study of *n* = 333 patients	About 75% experienced urine dipstick abnormalities or AKI. Patients who presented kidney dysfunction had higher mortality rates (11.2%) than patients without kidney involvement (1.2%)	Pei *et al* [88]
7	Observational study of *n* = 5449 hospitalized patients	The incidence of AKI was 36.6% with 14.3% of patients requiring dialysis. It was higher in patients admitted to the ICU. Patients with AKI had higher mortality compared to those without AKI (35% and 16.3%, respectively)	Hirsch *et al*[89]
8	*n* = 116 patients	Most of the patients with AKI were critical (52.4%) and incidence of AKI was 18.1% among patients admitted with COVID-19	Cui *et al* [90]

AKI: Acute kidney injury; COVID-19: Coronavirus disease 2019; ICU: Intensive care unit.

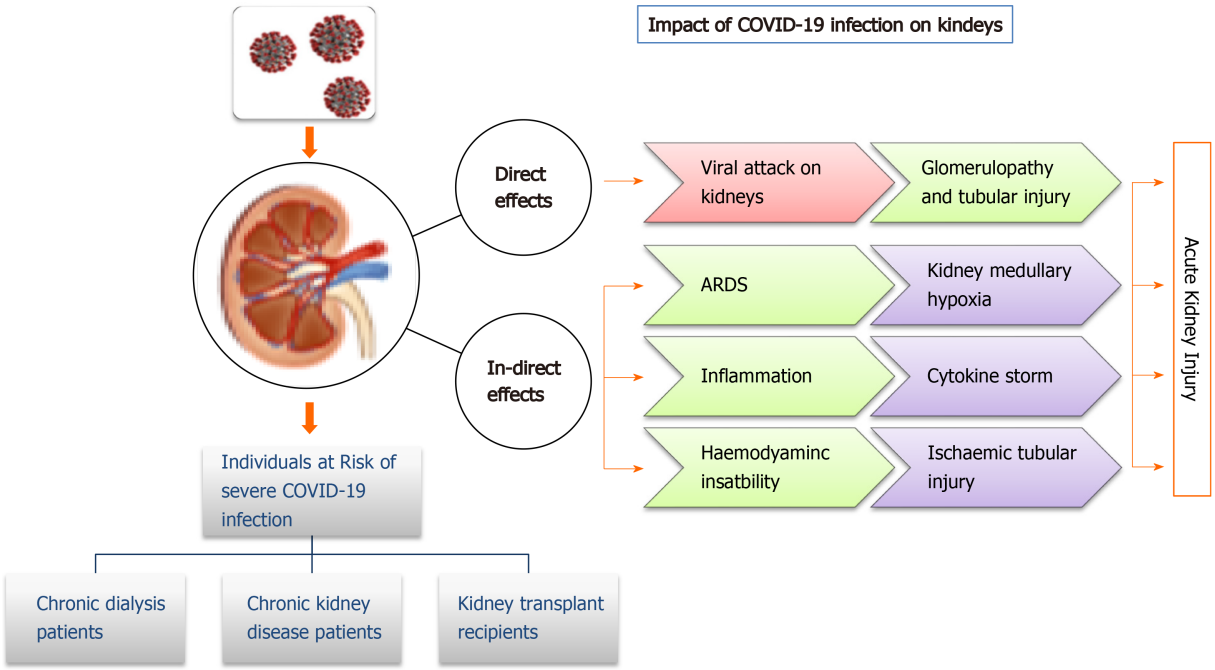


Figure 2 Kidney injury due to coronavirus disease 2019 attack. Direct and indirect effects of severe acute respiratory syndrome coronavirus 2 virus on kidneys. ARDS: Acute respiratory distress syndrome; COVID-19: Coronavirus disease 2019.

compared to other general SARS-CoV-2 infected patients[62]. However, this reduced or impaired inflammatory response may lead to a severe outcome of the disease. Thus, HD patients might need to be extra cautious and take all preventive measures to avoid contact with an infected person.

COVID-19 in kidney transplant recipients

To prevent graft rejection and to prevent inflammatory response, kidney transplant recipients are given immunosuppressants. Many countries with community transfer of COVID-19 have withheld transplantation procedures unless required in highly

selected cases when required as a life-saving procedure. Common symptoms of COVID-19 in transplant recipients include fever, cough, asthenia, myalgias and diarrhoea[63]. In different studies on COVID-19 infection in transplant patients showed numerous radiopacity and patchy shadows on chest radiographs[64-66], while in another study on 15 kidney recipients, 33% had no acute radiographic findings (Columbia University Kidney Transplant Program)[67].

Complete withdrawal of immunosuppressants such as calcineurin inhibitors is not recommended in COVID-19 patients in the absence of pneumonia, according to the European Renal Association-European Dialysis and Transplant Association guidelines [68]. However, reduction in its dose and withdrawal of mycophenolate, azathioprine or mTOR-inhibitors can be considered based on the severity of infection. Also, in such cases the prescription of antiviral and anti-inflammatory drugs should be done with caution, considering their drug-drug interactions with immunosuppressants[68]. Sudden withdrawal of immunosuppressants to clear viral load may lead to immune reconstitution and kidney rejection.

POST COVID-19 RECOVERY

Most people who get infected by SARS-CoV-2have mild or moderate symptoms and can recover completely after supportive medical care. Whereas some people, especially the elderly population with weaker immune systems and comorbidities, might suffer from severe symptoms and do not survive[69]. Currently there are no drugs licensed for the treatment or prevention of COVID-19. As treatment with several different drugs is under trial, the WHO is keeping a close watch on the efforts to develop medicines to treat COVID-19. In such cases, misuse of several drugs can lead to serious side effects and complications[70]. Meanwhile, non-pharmaceutical measures such as personal hygiene like hand washing and face masks, environmental sanitizing, social distancing and community-based decisions such as closure of schools, pubs, workplace restrictions *etc.* are followed at various levels to contain the infection spread.

The majority of people infected with SARS-CoV-2 make a complete recovery in 2-3 wk. Inconclusive studies are available on how long a person remains infectious after the symptoms of infection vanish. Doctors and medical experts across the globe are putting their best efforts to fight this novel coronavirus pandemic.With every passing day new information about its early symptoms, spread, variants, possible treatment strategies and post recovery complications is being added. Still there is no definite answer to the long-term implications of COVID-19. However, some emerging data point out that people suffer from shortness of breath, fatigue, joint pain and headache post recovery from infection[71]. Although, a patient's recovery may happen in about 2 wk of medical care, people may suffer from other ailments of the kidney, lungs and heart post-recovery. This condition is referred to as 'post COVID-19 syndrome' or 'long COVID19', which occurs due to persistent symptoms or delayed long-term complications beyond 4 wk. Older aged people, especially those with comorbidities, are more likely to experience lingering COVID-19 symptoms. Nalbandian *et al*[72] recently summarized the epidemiology and organ-specific sequelae of post-acute COVID-19 and the management considerations needed for interdisciplinary comprehensive care of such patients.

Most of the human viral diseases such as influenza virus do not produce a stable immune response. Understanding the immunity development and its long-lasting effects post SARS-CoV-2 infection needs systematic large-scale studies. Conclusive data on post infection immunity against COVID-19 is lacking. However, antibodies present post recovery would confer to immunity against re-infection, atleast temporarily. During recovery from COVID-19, immunoglobulin (Ig) M and IgG antibodies develop within days to weeks from the onset of symptoms[73,74]. However, there is not a clear and direct relationship between detectable antibody response and clinical improvement in a patient's condition[73,74]. Viral load peaks during the early stage of infection and then gradually declines as antibodies develop during the subsequent 2-3 wk[74,75]. The stability of neutralizing antibodies, primarily IgG, against COVID-19 is yet to be confirmed. During infection with SARS-CoV, IgG remained high for over 4-5 mo and subsequently declined during the next 2 years to 3 years[76]. It persisted upto 34 mo in patients recovered from Middle East respiratory syndrome-CoV[77].

There have been reports from different parts of the world about re-infection, possible by a distinct molecular form of the same virus[78]. A few studies have shown

that patients who had mild symptoms of COVID-19 developed a weaker and shorter immune response and show a decrease in antibody levels after 2-3 mo of infection[79, 80]. After almost a year of the COVID-19 pandemic, there is no consensus regarding persistence of neutralizing antibodies and re-infection by SARS-CoV-2.

CONCLUSION

COVID-19 or SARS-CoV-2 infected patients with pre-existing liver and kidney comorbidities are likely to have a poorer clinical prognosis. Such patients are at high risk of severe infection, and the mortality rate is higher compared to that of the general population. They should take utmost precautions to avoid contact with infected persons. SARS-CoV-2 infection can adversely affect liver and kidney functions in healthy individuals. Data available so far indicates that SARS-CoV-2 causes acute kidney and liver damage. However, the extent of damage and long-term consequences are yet to be elucidated. Currently in the absence of specific therapy for this viral infection and newly developed vaccines being unavailable to the majority of populations worldwide, further clinical studies are needed to understand COVID-19 pathology in relation to liver and kidney damage. This could further help clinicians to modify therapeutic approaches for better management of SARS-CoV-2 infection.

ACKNOWLEDGEMENTS

We acknowledge and thank all clinicians, medical staff and researchers who collected the clinical data of COVID-19 patients, which is very helpful in further understanding and formulating strategies to deal with this pandemic.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 June 28; 9(3): 234-256

DOI: 10.13105/wjma.v9.i3.234

ISSN 2308-3840 (online)

REVIEW

Environmental pollution and diabetes mellitus

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Author contributions: Helal M and El-Sikaily A wrote the paper and revised the paper.

Conflict-of-interest statement: The authors have no conflict of interest.

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Manuscript source: Invited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: Egypt

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0

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Abstract

Diabetes mellitus (DM) is a chromic metabolic disease that affects a large segment of the population worldwide. Physical inactivity, poor nutrition, and genetic predisposition are main risk factors for disease development. In the last decade, it was clear to the scientific community that DM development is linked to a novel disease inducer that was later defined as diabetogenic factors of pollution and endocrine disrupting agents. Environmental pollution is exponentially increasing in uncontrolled manner in several countries. Environmental pollutants are of diverse nature and toxicities, including polyaromatic hydrocarbons (PAHs), pesticides, and heavy metals. In the current review, we shed light on the impact of each class of these pollutants and the underlined molecular mechanism of diabetes induction and biological toxicities. Finally, a brief overview about the connection between coronavirus disease 2019 and diabetes pandemics is presented.

Key Words: Diabetes mellitus; Glucose; Heavy metals; Polyaromatic hydrocarbons; Pesticide; COVID-19

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Core Tip: In the review we summarize the relationship between different environmental pollutants of polyaromatic hydrocarbons, pesticides and heavy metals and their association with prevalence of diabetes mellitus. Further discussion of the molecular mechanisms of these pollutants that underline diabetes pathophysiology. Finally, a brief discussion on the association of diabetes mellitus, coronavirus disease 2019 and their clinical complications.

Citation: El-Sikaily A, Helal M. Environmental pollution and diabetes mellitus. *World J Meta-Anal* 2021; 9(3): 234-256

URL: https://www.wjgnet.com/2308-3840/full/v9/i3/234.htm

Grade D (Fair): D
Grade E (Poor): E

Received: January 28, 2021
Peer-review started: January 28, 2021
First decision: February 25, 2021
Revised: March 17, 2021
Accepted: June 3, 2021
Article in press: June 3, 2021
Published online: June 28, 2021

P-Reviewer: Acquaviva R, Moreira TMM, Sun X
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Li JH



DOI: https://dx.doi.org/10.13105/wjma.v9.i3.234

INTRODUCTION

The metabolic disease burden is mounting globally with accelerating rate[1]. Diabetes is a chronic metabolic disorder that significantly increases global disease burden and associated mortality[2]. Around 350 million individuals are suffering from diabetes worldwide, and epidemiological studies expect that there would be up to 590 million sufferers by 2035[3]. Diabetes costs the medical sector an estimate of 1.31 trillion dollars of the global domestic product[4]. Diabetes is the 7th leading cause of death worldwide. The disease is characterized by a deficiency in insulin secretion, hyperglycemia, and insulin receptor insensitivity[5]. Secondary diabetic complications include ulcer foot, renal failure, vision impairment, and cognitive dysfunction which can be developed over a prolonged unrecognized hyperglycemia[10]. It had been confirmed for decades that diabetes is a disease of obesity, poor dietary habits, smoking, and physical inactivity[6-8] but a decade ago it was proposed that environmental pollutants can be a potent inducer to diabetes mellitus (DM)[9]. Furthermore, due to widespread industrialization, a large segment of the world population is increasingly exposed to elevated concentration of environmental pollutants[5].

Diabetes can be of type I (type I DM, T1DM) or type II (type II DM, T2DM). T1DM results from pancreatic β-cell destruction due to reactive oxygen species (ROS) overload or autoimmune self-destruction[11]. Molecular mediators of T1DM include, but are not limited to, ROS, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon γ (INF γ)[12,13]. On the other hand, T2DM results from either reduction in insulin synthesis and secretion or insulin resistance as the main hallmark of the disease[14,15]. The burden of T2DM arouses in its associated complications affecting more than 400 million people worldwide and is increasing annually[16].

Environmental pollution is defined as the introduction of harmful substances to human into the environment. Pollutants can be solid, liquid, or gaseous in nature, and elevated concentrations of any form will affect the quality of our environment and living organisms[17]. The term diabetogen was firstly introduced by Campbell[18] in a letter submitted to the *British Medical Journal* describing his finding that excess consumption of mustard oil was the cause of increased incidence of diabetes among Natal Indians. Later, he postulated that diabetes was developed as a result of chemical chelation of sulfhydryl group that can interfere with carbohydrate metabolism. Recent reports demonstrated that environmental pollutants and endocrine disruptive chemicals can be attributed to the increased number of diabetes sufferers among different populations[7,19-24]. Following this, obesogenic chemicals, such as polychlorinated biphenyls and pesticides, also arouse on the front line of research[25].

Environmental pollutants are diverse examples of heavy metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, and different dioxins. They have various health impacts on the biological systems. Recently, there is a call for minimizing human exposure to such pollutants in order to improve human health and decrease economic, coast, and disease burden worldwide[24]. Dioxins are a group of highly toxic persistent organic pollutants (POPs), released in the environment as industrial by-products. They comprise molecules such as polychlorinated dibenzo-p-dioxins, polychlorinated/polybrominated biphenyls, and polychlorinated dibenzofurans[26].

KREBS CYCLE, GLYCOLYSIS, AND BLOOD GLUCOSE LEVEL

Glycolysis is the process of converting glucose into pyruvate/lactate following cellular uptake of glucose and glucose phosphorylation. Adenosine triphosphate (ATP) and energy required for glycogenesis and lipogenesis are also provided through glycolysis. Different cells utilize glycolysis through different regulation levels such as glucose phosphorylation, glucose uptake, and/or fructose-6-phosphate conversion into fructose-2,6-biphosphate (F2,6p2). Moreover, glycolysis rate is strictly regulated by various factors such as glucokinase (GK), glucose transporter-4 (GLUT-4), and 6-phosphofructo-1-kinase (6PFK1). Briefly, 6PFK1 activation occurs *via* F2,6p2 which is a potent activator to 6PFK1 and regulation of glycolysis rate. F2,6p2 production and breakdown are achieved through 6-phosphofructo-2-kinase/fructose-2,6-biphosphate (6PFK2/FBPase2) in different cells in response to nutritional variations in the environment[27].

Importantly, it has been demonstrated that glycolysis is closely associated with different aspects of integrative physiology, in particular, glucose production in liver, insulin secretion in the pancreas[28-30], muscle glycolysis[31], adipose tissue lipogenesis[32], and hypothalamus glycolysis[33]. Furthermore, liver glucose entrance and clearance are achieved through cycles of glycolysis and glycogenesis regulating blood glucose level[34]. Mechanistic interaction between insulin and glucose is mainly additive or synergistic for stimulating GK dissociation in hepatocytes from the inhibitory GK dissociation from its inhibitory binding to GK regulatory protein in order to improve the hepatic expression of GK and 6PFK2/FBPase2[35].

Linking glycolysis as a physiological process with diabetes can obviously be seen from the fact that decreased or dysregulated glucose phosphorylation is a glycolytic critical step contributing to elevated HGP and developed hyperglycemia[27].

Several cross-sectional and prospective studies reported the relation of blood sugar level, sugar-related metabolites, glycolysis/glucogenesis components, and tricarboxylic acid (TCA) pathway intermediates with prediabetic syndrome, insulin resistance, and diabetes[36-39]. TCA cycle is the main source of energy in cells by yielding ATP. TCA is also known as citric acid cycle or Krebs's cycle. In addition, TCA works as a precursor pathway for gluconeogenesis during fasting[40].

Among different glycolysis/glucogenesis metabolites, lactate (the endpoint of anaerobic glycolysis) showed a differential response change in its concentration during oral-glucose-tolerance test (OGTT)[41]. Moreover, lactate metabolite was a relevant predictor for T2DM incidence in some epidemiological studies[42-44]. Although TCA metabolic cycle and glycolytic pathway were altered in pancreatic β-cell lines[45], it is unlikely that lactate/pyruvate circulated in the blood directly influence insulin secretion given that lactate/pyruvate transporter monocarboxylate transporter 1 is inhibited in the β cells responsible for insulin resistance and metabolic phenotypes [46]. Plasma lactate concentration was increased upon 75-g OGTT and hyperinsulinemic-euglycemic clamps in insulin resistant objects compared to insulin sensitive objects[47-49]. In addition, there was an observed link between circulating lactate and glucose homeostasis as lactate is a precursor of hepatic gluconeogenesis enhancing the endogenous glucose production. Finally, the importance of plasma lactate in metabolism lies in its utilization as the major carbon source to mitochondrial TCA mostly in peripheral tissues[50,51].

Among diabetes clinical features, peripheral insulin resistance is a major concern where the exact molecular mechanism has not been elucidated in detail yet[52]. Researchers have described that diabetic myotubes derived from T2DM individuals experience increased basal glucose and lipid oxidation[53]. These alterations in myotubes physiology were attributed to reduced TCA cycle flux[54]. This was confirmed by TCA cycle inhibition by malonate (competitive inhibitor of TCA cycle enzyme succinate dehydrogenase) which resulted in a decline in acetate oxidation and full palmitate oxidation[55]. TCA cycle flux reduction can be responsible in part in the diabetic phenotype. An explanation to this lies in the fact that acetate, malate, citrate, pyruvate, glutamate, and isocitrate (TCA metabolites produced by anaplerosis process)[56] enter TCA cycle at different sites before their oxidation occurs which powers the conversion of ADP to ATP. The generated energy is transferred to electron transport chain complex I[57]. It was found that mitochondrial ATP production was reduced in diabetic patients due to impaired oxidative phosphorylation[58,59]. Other studies could not confirm this finding based on transcriptomic profile of diabetic myotubes[60]. Insulin resistance in type II diabetic patients has been connected to increased oxidative stress, DNA damage, and lipid and protein damage[61].

Conventional laboratory tests can be utilized for confirming the reality of toxin exposure inside animals' bodies such as γ-glutamyl transferase which is an enzyme that recycles glutathione for POPs detoxification[26], determination of pollutant metabolites in urine by using gas chromatography mass spectrometry[62-64], complete blood counts, alanine transaminase, and high-sensitivity C-reactive protein.

PESTICIDES

Pesticides are a group of natural or chemical compounds utilized in the agriculture field to fight insects and enhance plant growth[65]. Pesticides are receiving more attention recently because they are the most commonly used compound nowadays with more than 2.3 billion kgs used worldwide[66]. Pesticides include both organochlorine (OC) and organophosphorus (OP). OP pesticides can degrade easily and have a short half-life cycle. Hence, most studies related to OP are designed to comply with

their nature[67,68]. OP pesticide exposure was associated with diabetes prevalence in two different Pakistani and Cameroonian populations through dysregulation of pancreatic function of β cell[69].

Data linking pesticides and diabetes is scare, and more research and epidemiological studies are needed[70]. The first reported and largest systematic review of observational analysis linking diabetes association and pesticides was conducted by Evangelou *et al*[71]. In their study, they summarized the finding of their analysis as there is an association between OC (chlordane, trans-nonachlor, oxylchlordane, heptachlor, DDE, DDT, HCB, and dieldrin) and T2DM.

In a large-scale population study applied on 33457 pesticide applicators in the US initiated by Agriculture Health Study, an association was found between OC compounds, such as chlordane, aldrin, dichlorvos, heptachlor, alachlor, cyanazine, and trichlorfon, and diabetes[72]. When the study was extended to include wives of those pesticide applicators, more diabetic linked compounds were identified such as fonofos, phorate, and parathion (organophosphate) and one OC as dieldrin[73]. Furthermore, pesticides (mevinphos, endosulfan, carbaryl/sevin and benlate) as a major agriculture chemical waste were reported to be significantly associated with diabetes occurrence among Thai farmers[74]; the same finding was reported in Korean farmers in a cohort study comprising 2559 farmers[75]. Egyptian diabetic children showed a correlation between CD4%, CD4/CD8 ratio, and HLADR% and lindane and malathion (organophosphate compound), highlighting the need for further studies to reduce pesticide use in Egypt[76]. A US large-scale screening of pesticide applicators of non-Hispanic white males found that chlordane, aldrin, and heptachlor pesticides specifically correlated with increased diabetes risk[72]. Moreover, diabetes-associated mortality was higher in Australian individuals exposed to pesticides than the general population[77]; the same finding was reported among Indian farmers[78]. Finally, in a recent and pioneering study, OCs compounds (chordane, trans-nonachlor, p,p'-DDT, β-HCH, *trans*-chordan, and mirex/kepone) were detected in sera of more than 75% of Chinese participants in a nationwide Chinese study. It was reported that the associated mechanism may be disrupted lipid metabolism, elevated hemoglobin A1c, triglycerides, and lactate dehydrogenase[79].

A recently published research article studied the diabetogenic impact of dichlorvos and atrazine in Drosophila melanogaster. In this study, the authors utilized a fly model with conserved insulin/insulin growth factor-like signaling (IIS) and exposed the embryonic stages to various concentrations of either the pesticide (dichlorvos) or the herbicide (atrazine). Dichlorvos induced T1DM, while atrazine induced T2DM. The associated mechanism included excess oxidative stress, altered JNK, and AKT phosphorylation[80]. Acute and chronic rat exposure to dichlorvos caused hyperglycemia associated with UDP glucose synthesis inhibition that was reversed by insulin administration[80].

Secondary diabetic nephropathy is a common secondary manifestation of diabetes. Various pesticide compounds were detected in sera samples of individuals with chronic nephropathy. It was found that the six chemicals were *p,p'*-DDT (dichlorodiphenyltrichloroethane), *p,p'*-DDE (dichlorodiphenyltrichloroethylene), beta-hexachlorocyclohexane, oxychlordane, trans-nonachlor, and heptachlor epoxide to be loaded excessively in their blood sera. It was also demonstrated that androstane receptor/pregnane X receptor and aryl hydrocarbon receptor pathway are the main indulged pathways in diabetic secondary manifestation[81].

Trichlorfon insecticide is broadly utilized in aquaculture farms for reducing fish parasitic infection. Silver cat fish *Rhamdia quelen* exposed to an acute dose of trichlorfon exhibited reduced activity of creatine kinase and complexes II-III and IV in muscle, lower content of polyunsaturated fatty acids (Omega 3 and Omega 6), and altered fatty acid profile in comparison to the control group[82].

Nonobese diabetic mice (NOD) mice exposed to chronic high dose of DDE suffered significantly from T1DM development and hyperglycemia because of the irregular activity of T-cell and suppressed cytokine secretion such as IL-4 and IL-10[83].

Gestational DM (GDM) is a form of diabetes that develops during pregnancy affecting 1%-14% of the United States population[84]. GDM is considered a predisposition for T2DM development. The main risk factors for GDM include older maternal age, obesity, previous macrosomic infant, and diabetes history[85]. Wistar rats exposed to imidacloprid (IMI) and chlorpyrifos (CPF) during gestation resulted in striking results where female rats developed diabetes and their offspring also developed diabetes over the long term of life indicating intergenerational adverse effect. Diabetes developed in those Wistar rats showed signs of increased fasting blood glucose, decreased GLUT4 and NFkβ, and reduced high-density lipoproteins (HDL) [86].

Carbendazim (fungicide) administration to mice for 14 wk caused elevation in the levels of triglycerides in their feces as an indication of dysregulated lipid absorption in intestine[87]. Pesticide may have an effect on energy absorption in intestine by regulating gut microbiota[88]. Evidence of rat exposure to pesticides showed a deteriorative effect on pancreatic β cell by imazamox (36 mg/b.w.) treatment[89] and change of pancreas size and glucose homeostasis by IMI (1 mg/kg) treatment[90]. Meanwhile, rats treated with dimethoate at dose of 200 mg/kg b.w. showed signs of pancreas fibrosis and profibrotic cytokines such as transforming growth factor β1[91]. Pesticides affected not only pancreas cytotoxicity but also hormone secretion. Malathion treatment at 400 ppm resulted in an elevated secretion of insulin[92]. On the other hand, both amitraz (10 μmol/L)[93] and diazinon (60 g/kg b.w.)[94] reduced insulin secretion in rat pancreas. Furthermore, pesticide mixture of pirimicarb, quinoclamine, thiram, and ziram in rats led to overexpression of glucagon in pancreatic α cells which is an indicator of increased glucogenesis[95].

Insulin resistance and dysglycemia are also associated with pesticides exposure. Permethrin, CPF, and IMI treatment in mice led to an elevation in blood glucose level [96-98]. Additionally, obesogenic effects of pesticides have been documented in different studies. CPF treated rats (3 mg/kg b.w.) had increased body weight without affecting animal appetite or food consumption[99]. Opposite effect was noticed when pesticide mixture of pirimicarb, quinoclamine, thiram, and ziram treated rats led to decreased body weight[95].

Several mechanisms were proposed explaining the effect of pesticides on diabetes [68]. OC pesticides have variable molecular weights and cellular targets which imply that not a single mechanism can define their mode of action. The underlying mechanisms of T2DM development include adipose tissue inflammation, liver lipotoxicity, ectopic lipid accumulation in liver, pancreas, and muscle, and finally mitochondria dysfunction[100]. *In vivo* study indicated that rats fed salmon oil enriched with OC pesticides had insulin resistance, dyslipidemia, visceral obesity, and non-alcoholic inflammatory fatty liver[101]. Oxychordane, trans-nonachlor, and p,p'-DDE exhibited strong inhibitory effect on pancreatic β-cell dysfunction[101], decreased cell metabolism, and induced obesity[102] or affected insulin release and action and dysregulated glucose homeostasis[103] or increased oxidative stress and inhibited cholinesterase activity[104]. Humans' OC background exposure (long term-low dose) alongside their lipophilicity and hydrophilicity and resistance to metabolic degradation indulges them into tissue toxicity and bioaccumulation through lifetime [100].

So far, the results of different studies demonstrated a contradictory finding regarding the exact effect and firm association between different classes of pesticides and incidences of different classes of diabetes. Further studies are still in need to decipher the exact mechanism and underlying effector[105].

PAHS

PAHs are a group of lipid soluble environment-related pollutants that are characterized by the presence of aromatic ring in their chemical structure. They are generated from the incomplete combustion of organic materials including wood, gasoline, and coal during cooking process of contaminated food and industrial production processes [106-108]. PAHs include several members such as naphthalene (NAP), fluorene, anthracene, phenanthrene (PHEN), fluoranthene, pyrene, benzo[a] anthracene, chrysene, benzo[a]pyrene (BaP), benzo[b] fluoranthene, benzo[e]pyrene, benzo[j]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, indo[1,2,3-cd]pyrene, and dibenzo[al]pyrene[26].

PAHs are ubiquitously toxic with high melting and boiling points and low vapor pressure[109]. PAHs are listed among the top ten priority list of hazardous materials according to their characters[110]. They enter the human body *via* food consumption, cigarette smoking, and aquatic and air pollution[17,64,111-113]. Once inside the body, they are metabolized into mono hydroxylated PAHs (OH-PAHs) and excreted *via* urine route. Thus, it is anticipated to consider urinary metabolites of PAHs as a good biomarker for environment pollution associated with certain diseases[114].

One of the earliest studies to investigate the association between urinary metabolites of PAHs in non-Western population was done by Nam and Kim[115]. In a national wide scale study in Korean population, it was found that the urinary metabolites of PAHs were closely associated with diabetes prevalence in men and women (diabetes prevalence was 6.5% among participants). The most commonly diabetic associated

PAHs were 2-naphthol (2-NAP) and 2-hydroxyflourene with geometric mean value of 4.11 and 0.45, respectively, in men, while 2-NAP was only associated with diabetes development in women with geometric mean value of 1.81[115]. Another retrospective study among Chinese population indicated a dose-dependent association between urinary OH-PAHs and prevalence of diabetes especially in female participants[111]. In addition, another cohort study among Chinese cooking females in Wuhan demonstrated that female exposure to OH-PAHs and fraction exhalation of nitric oxide during cooking were positively associated with diabetes prevalence among them[107]. An elegant study was published recently to investigate the association between OH-PAHs and incidence of DM development by analyzing the reported research output in different databases like Scopus, PubMed, and Web of Science. Meta-analysis of more than 668 research articles reported a significant association between diabetes and urinary NAP, total OH-PAH, and fluorine (FLU)[62].

Male Kunming mice that received different doses of PHEN for 28 wk exhibited elevated levels of insulin resistance and hyperinsulinemia as interpreted by homeostasis model assessment-insulin resistance (HOMA-IR) and HOMA β cell. Moreover, insulin receptor signaling pathway was inhibited in skeletal muscles, accompanied with glucose intolerance and a disruption in adipocytokine secretion[116]. The effects of PHEN can also extend to offspring until adult stages. Maternal exposure to PHEN during mice gestation period disrupted glucose homeostasis machinery and caused diabetes in adult stages of their offspring[117].

Normoglycemic (normal glucose level) and diabetic rats induced by intraperitoneal injection of streptozotocin (STZ) when treated with BaP exhibited increased kidney weights and blood urea nitrogen compared to the other group treated with STZ only. Authors concluded that BaP increased kidney weight and renal injury as a secondary complication to DM[118]. Resveratrol drug could attenuate BaP-induced pancreatic β-cell dysfunction by decreasing oxidative stress and apoptosis in those cells[119].

Several urinary OH-PAHs of 2-hydroxynaphthalene, 2-hydroxyfluorene, 9-hydroxyfluorene, and 2-hydroxyphenanthrene showed a positive correlation with diabetes occurrence among adults in the United States[120]. Single OH-PAHs have been reported to be connected with diabetes prevalence in different studies. NAP metabolites, 1-NAP[121,122] and 2-NAP[121,123], were found to be significantly associated with diabetes in western populations. FLU metabolites, 2-FLU, 3-FLU, and 9-FLU, were also strongly correlated with diabetes occurrence[111,120,121]. In addition, PHEN metabolites, 1-PHEN, 2-PHEN, 4-PHEN, and 0-PHEN, were reported to be conjugated with increased risk of diabetes occurrence[63,111]. Furthermore, pyrene metabolite, 1-OHP, was found to be significantly associated with diabetes in the same reported studies[111,121,124].

The exact mechanism by which PAHs induce diabetes is under investigation; however, it has been proposed that PAHs exposure can interrogate either insulin sensitivity or decreased insulin production[26] through modulating oxidative stress, systemic inflammation, and induction of pancreatic β-cell dysfunction[63,122,125-127]. Some reports indicated that PAHs can induce either a systemic disturbance in the antioxidant system leading to elevated oxidative stress[128] or increased inflammation and release of proinflammatory markers such as CRP and IL[122]. Furthermore, disruption of the endocrine system is a main theme of PAHs-induced diabetes resulting in an increase in estrogen hormone level[129].

HEAVY METALS

Heavy metals are a group of chemical elements of high atomic weight and superior toxicity at low concentrations[130]. They originate either from natural sources of volcanic eruption and metal-bearing rocks or from anthropogenic sources of various agricultural and industrial activities. Both sources potentiate heavy metals concentration in the environment due to their persistence nature causing a public health problem affecting more people worldwide[131]. Heavy metal pollution has increased tremendously in developing countries[1], and increasing body of evidence linked heavy metal toxicity with hepatic, renal, neurological, and hematological adverse health problems[132].

Metals can be categorized into toxic metals, such as arsenic (As), Pb, cadmium (Cd), and Hg, and essential metals, such as copper (Cu), chromium (Cr), nickel (Ni), Se, and cobalt (Co), which are also metalloestrogens, and both kinds increase cardiovascular and diabetic risks[133].

Heavy metal load in the body exaggerates the progression and severity of DM by altering islet function of the pancreas. Toxic metals either directly disrupt glucose uptake or interfere with glucose regulation leading to development of T1DM or T2DM [5]. Exposure to heavy metals occurs through ingestion of contaminated food, drinking water, and ambient air[134]. In terms of heavy metals toxicity, it should be mentioned that single heavy metals have different rates of toxicity on pancreatic islet cells[135, 136]. Some trace metals under specific concentrations exert various biological functions in which glucose hemostasis is involved[137], and any imbalance in its concentration will impact pancreas biology and insulin synergy production leading to dysglycemia [138].

Metals are involved in many biological aspects of muscle contraction, nerve impulse, and kidney function. They may either function as prosthetic groups of many proteins or be an integral part of other proteins as metalloproteinase/metalloenzymes [139,140]. Diet with low content of nutritive essential metals forces the body to absorb and make use of toxic metals instead. Body substitution of scare essential metals with available toxic metals [zinc (Zn), iron, and potassium] will disrupt various endocrine pathways[141]. Abundance of metal toxicity in the body interferes with the physiological functions of the body[136]. For example, Zn deficiency induces the body to absorb more Fe and use it instead of Zn[142].

Cd, Ni, As, and mercury are examples of toxic metals that induce pancreatic cell death, alter pancreas function, and increase diabetes risk. On the other hand, Co, lead, manganese (Mn), mercury, and Cd were reported to be endocrine disruptive elements contributing to DM development[143,144]. Because of the rapid and uncontrolled industrialization in several countries, heavy metals concentration in the environment is rapidly increasing leading to elevated accumulation in the biological systems[5]. Up till now, the association of environmental exposure to different metals with diabetes development has not been fully covered[134].

Cd belongs to trace elements that occupy Earth's crust in concentrations ranging from 0.1 ppm to 0.5 ppm[145]. Cd toxicity in animals results from its accumulation in the internal organs of the animal and interruption of the common metabolic pathways in kidneys, lungs, bone marrow, pancreas, and liver[131,146]. Some studies could not prove a strong and significant connection between Cd and diabetes prevalence[147, 148], but other studies indicated that Cd exposure can increase the risk of T2DM development especially in older individuals above 50 years by increasing insulin resistance in their bodies[149,150]. Additionally, several studies have reported the significant association between Cd levels and T2DM[151-153] mainly through downregulation of glucose transporter protein GLUT4 and pancreatic β-cell dysfunction[5]. A recent review work was published and indicated an emerging role of Cd in the pathology of various diseases especially T2DM. Hence, Cd was regarded as a hyperglycemic metal that increases glucose levels in blood *via* decreasing insulin production mainly by β-cell destruction[154].

Chromium (Cr) biological activity is confined to regulation of glucose transporter (GLUT4) activity. Cr upregulates GLUT4 translocation into muscle cells which regulates insulin signaling pathway and metabolism[155]. It has been utilized for reducing diabetes risk and decreasing blood glucose level. Prolonged deficiency of dietary Cr may lead to diabetes[156]. Several studies assure the importance of Cr supplementation through diet in reducing oxidative stress, improving cardiometabolic function, and decreasing risk of DM development[157-159].

Iron (Fe) is another example of essential transition metal that is involved in the synthesis of hemoglobin and myoglobin proteins responsible for oxygen transport during respiration[160]. In addition, ferritin, the main protein responsible for Fe carriage, was found to elevate in newly diagnosed diabetic individuals[161,162]. Furthermore, it was found that women with gestational diabetes had higher index of iron dysregulated metabolism under pregnancy stress adaptive disorder[163]. Momeni *et al*[164] proposed that serum ferritin is a good indicator for diabetes development and response to therapy as its level increased and decreased, respectively, alongside HbA1c level. Other studies also supported this postulation assuring ferritin level as a surrogate marker for disease onset[165,166]. Iron overload in tissues led to hemochromatosis which was found to be tightly associated with T2DM in several studies[167-170]. About 11 case control and cohort studies described the association between serum ferritin level and soluble transferrin receptor-to-ferritin ration and DM while no significant association was found in case of serum ferritin receptor[171]. Meta-analysis study of hematological parameter indices and iron metabolism in T2DM patients showed that iron profile and total iron binding capacity were significantly higher in T2DM patients than normal individuals[172].

Zn is also a trace metal that is integrated in cellular processes of cell division and apoptosis. Zn importantly maintains the integrity of pancreatic β cells and can be used as a possible treatment of diabetes[5,173-175]. Fasting blood glucose level was found to be associated with urinary levels of Co, Zn, and Ni as a prognostic indicator[176]. T2DM patients have hypozincemia (lower serum levels of Zn) and hyperzincuria (urinary Zn levels)[177]. Decreased Zn serum level adversely affected the ability of pancreatic islet cells to synthesize and secret insulin[178-180]. A mutation in Zn transporter protein ZnT8 has been connected to T2DM development and progression [181]. Mitigation effect of Zn supplementation on relieving DM secondary complications and glucose homeostasis has been proposed in a number of different studies and meta-analysis cohort studies[182-186].

Ni belongs to ferromagnetic elements, silver-white in color and used in alloy manufacturing and production of Ni-Cd batteries[187]. Ni is widely distributed in nature as a mixture with other elements such as sulfur, As, and iron[188]. Ni concentration was found to be significantly connected with T2DM prevalence[176]. In a Chinese survey of adult male and female individuals, elevated concentration of urinary Ni was found among those with T2DM[189]. To the best of our knowledge, no cohort studies have been conducted to study its association with female gestational diabetes development[190].

As is a toxic metalloid that is naturally occurring as organic or inorganic form in the environment. It is mainly used in insecticide, herbicides, and pesticide production. It can be solubilized in ground water with a concentration up to 10 μg/L[191]. Chronic exposure to As is firmly connected with cancer development[192-194], vascular diseases[195,196], and neurological disorders[197]. As direct contact through diet and contaminated water was found to directly affect pancreatic β cell and decrease insulin production[198]. Available data on the connectivity between As and diabetes is not conclusive and the need for more accurate measurable studies is demanding[199]. Yet some reports highlighted that As-induced diabetes can be initiated through altering TNF-α, GLUT4, and mitogen-activated protein kinase[5]. Ramdas *et al*[200] indicated that pancreatic miR-2909 was significantly higher in NOD mice than control ones upon exposure to low dose of As. miR 2909 control expression of genes coding for pancreatic duodenal homeobox 1 and phosphatidylinositol-3 kinase (PI3k) regulated insulin synthesis and release. STZ diabetic mice model showed higher level of expression of trivalent inorganic As with consequent increase in As accumulation in their bodies and susceptibility to As-induced systemic toxicity[201]. In a Swedish children cohort study, researcher found that children with T1DM tend to have higher levels of aluminum and As metals which were traced back to their accumulation in their pregnant mothers too [202]. In a cross-sectional study in the United States covering 429 children and adolescents diagnosed with T1DM, they found higher level of As metabolites associated with higher odds of T1DM[203]. Furthermore, a Canadian community level screening indicated that higher levels of fluoride and As in drinking water were linked to higher T1DM incidence[204].

Magnesium (Mg) is known as the most important macronutrient essential for proper health maintenance through its integration with more than 300 enzymes in the body involved in nerve transmission, glucose homeostasis, and DNA and RNA synthesis[205-209]. Mg is another example of essential regulators of insulin-mediated glucose uptake in the body. A large prospective cohort study indicated an association between Mg diet level and T2DM development risk[210-214]. Dietary supplement of Mg was found to reduce T2DM risk[215]. A number of studies supported the dietary supplementation of Mg to enhance general health status and decrease risk of T2DM development[185,211,216,217].

Mn acts as a cofactor in several enzymes involved in the metabolism of carbohydrates, proteins, and lipids[218,219]. In addition, it is essential for proper utilization of vitamins (C and E), biotin, and choline[220]. More importantly, Mn is a cofactor of pyruvate carboxylase (enzyme indulged in conversion of non-carbohydrates compounds into glucose *via* gluconeogenesis), hence indirectly implicated in diabetes development as reported by Forte and his research group[177]. Dietary Mn was found to decrease inflammation and reduce risk of T2DM development in postmenopausal women[221]. Inclusion of Mn-enriched diet may decrease the risk of DM in several reported studies and cohort studies[222,223].

Copper (Cu) is crucial for various biological functions as it was found to be integrated in regulating activity of superoxide dismutase (free radical defense system) [224]. Excess Cu induces oxidative stress leading to β-cell dysfunction. Hence, abnormal Cu levels may accelerate DM development and associated complications [225]. Imbalanced Cu complications can result from its association with low-density lipoproteins and HDL imbalances due to elevated cholesterol level in the body[226].

Carbohydrates-enriched diet interferes with Cu metabolism and availability in diabetic patient sera[227]. On the other hand, in a large scale cohort study including more than 70,000 women, it was found that low dietary intake of Cu-Zn ration is associated with lower T2DM risk among women[228].

Cr and calcium (Ca) play a vital role in carbohydrate and lipid metabolism, thus controlling insulin synthesis and release *via* indirect route[137,155]. Co overload will increase the expression of GLUT-1 and inhibit gluconeogenesis[229].

Low levels of antimony, uranium, tungsten, and molybdenum were consistently associated with diabetes prevalence in the United States general population, while cesium, thallium, barium, and lead were not evidenced[141]. Antimony is used in ceramics, metal alloys, and fire-retardant[230]. Ingestion of large quantities of antimony not only induces substantial gastrointestinal effects including nausea, diarrhea, and vomiting but also increases diabetes risk[231]. Uranium is commonly used in munitions, and the studies linking urinary uranium levels with diabetes are limited[232,233].

A nationwide association study on US midlife women showed that there was a higher urinary concentration of lead, As, and Zn in diabetic high-risk group than normal group[134].

Single heavy metals can increase the risk of T2DM development through various mechanisms. First, some toxic metals (Pb, Hg, As, and Cd) can increase the oxidative stress burden in the pancreas by increasing the concentration of free radicals (hydrogen peroxide, superoxide, and nitric oxide)[234]. Alongside the weak biological ability of pancreatic islet cells of antioxidant defense system and their high expression of metal transporter proteins, islets cells become extremely sensitive and rapidly deteriorate resulting in cell damage and apoptosis[5]. Second, heavy metal-induced oxidative stress was found to be firmly connected with reduced insulin gene activity and insulin mRNA expression in β cells of the pancreas[235,236]. The change in pancreas gene expression activity was found to be accompanied with insulin receptor impairment, glucose uptake dysfunction, induced gluconeogenesis, hepatic glycolysis, and pancreatic glucan secretion[237,238]. Third, some heavy metals at certain concentration play a key role in glucose hemostasis as they are part of the metabolic pathway of glucose, cofactors in glucose hemostasis, and pancreatic β-cell function[5,137]. Finally, toxic heavy metals (Cd, Pb, Hg, and As) and essential elements (Cu, Cr, Se, Ni, and Co) can act as endocrine disruptive agents *via* interfering with PKB/AKT-mediated GLUT4 expression *in vitro*, glucose hemostasis, and disruption of lipogenesis [5,231,239,240].

The curative characters of some trace elements to antagonize the toxic effects of toxic metals are very crucial in keeping the integrity and biological function of the pancreas for the prevention of DM development[241]. Additionally, it was demonstrated that hyperglycemia led to abnormal metabolism of trace elements and associated proteins in various organs[151,242-246].

Furthermore, deficiency or overload of heavy metal concentration in the pancreas can cause pancreatic cell dysfunction. For instance, Zn at normal levels plays an important part in pancreatic cell function, insulin synthesis, storage, and secretion[136,175]. On the other hand, Zn deficiency decreases insulin synthesis and secretion[247], while its overload leads to oxidative damage and cellular toxicity[248,249]. Decreased insulin sensitivity, glucose intolerance, and mitochondrial distortion of pancreatic acinar cells are remarkable examples of Cu deficiency[250]. For selenium, high level of selenium was found to be a strong diabetogenic inducer possibly through impairing insulin responsiveness, increasing glycolysis, and glucagon release stimulation resulting in hyperglycemia and obesity[251,252].

DM *VS* CORONAVIRUS DISEASE 2019

The newly surged coronavirus disease 2019 (COVID-19) is a respiratory infectious disease that was first detected in Wuhan city in China[253]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to positive single stranded RNA viruses naturally mutated genetically to infect human through its intermediate hosts (pangolins and bats)[254,255]. Clinical symptoms of COVID-19 patients include dyspnea, lymphopenia, severe respiratory distress, cytokine storm (awry and excessive uncontrolled), and blood micro-clotting in lungs and different body organs [256-258].

Diabetic individuals are at high susceptible risk of COVID-19-induced adverse outcomes and medical complications[259,260]. For instance, the early reports from Chinese hospitals in Wuhan reported that people with diabetes admitted to ICU due to

COVID-19 contradiction had greater rates of mechanical ventilation and death rates than non-diabetics[261]. On the other hand, other reports did not support this finding, such as the study by the multicenter French Coronavirus SARS-CoV-2 and Diabetes Outcomes (CORONADO)[262] and that in the United Kingdom[263].

Poor glycemic control is reported to be a high-risk factor for death-related mortalities among hospitalized COVID-19[264,265]. Interestingly, COVID-19 can induce hyperglycemia in patients without diabetes and induce elevated blood glucose level[266]. Diabetes-associated comorbidities include hyperglycemia, nephropathy, hypertension, obesity, and dyslipidemia[267], which predispose them to poorer and decreased effective medications. Women infected with COVID-19 with developed gestational diabetes can develop severe COVID-19 infection; however, the study results are not confirmed and need further analysis[268,269]. The toxic impact of elevated glucose titer in blood affects various cellular pathways collectively known as glucotoxicity[270]. Although it was not confirmed conclusively, it was suggested that COVID-19 induces tissue injuries within the diabetic milieu. Codo *et al*[271] reported that DM favored COVID-19 infection *via* tissue hypoxia and increased angiotensin II, fibrinogen, D-dimer, insulin resistance, and oxidative stress[272,273]. Concurrent tissue damage in several organs due to COVID-19 infection and DM subject those patients to maximum risk of mortality[260].

Both COVID-19 and diabetes pathways intersect in a number of common pathologies of inflammation (acute *vs* chronic, respectively), tissue damage (acute *vs* slowly progression, respectively), hyperglycemia, hypercoagulation, endothelia dysfunction, and tissue fibrosis[259]. The multifactorial nature and pathway complexity of both COVID-19 and DM make those patients in need of urgent critical care to increase their survival outcome[274].

CONCLUSION

Recent report over the last decade implied the diabetogenic effect of different environmental pollutants. Each category has its own mechanism of induction, and link was further confirmed by an array of different cohort and meta-analysis studies by several researchers all over the world. Pesticides, PAHs, and toxic heavy metals are all implicated in the progression and severity of DM mostly through dysregulated antioxidant machinery, elevated oxidative stress in pancreatic β cells, interferences in insulin synthesis, storage, and release from pancreas. On the other hand, dietary supplementation of some metals, such as Mg, Mn, and Zn, has been shown to decrease risk of DM development. Further studies are inevitably demanding to achieve several goals as follows: (1) Different population assessment of their history and current diabetic status in order to generate an up-to-date informatics on disease prevalence and spread; (2) Dissecting the exact molecular mechanism and identifying the molecular players included in disease development; and (3) Finally, introducing novel therapeutic regimes to treat the disease and its associated complications.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 June 28; 9(3): 257-276

DOI: 10.13105/wjma.v9.i3.257

ISSN 2308-3840 (online)

REVIEW

Immune response to *Helicobacter pylori* infection and gastric cancer development

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution

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Abstract

Gastric adenocarcinoma is a global health concern, and *Helicobacter pylori (H. pylori)* infection is the main risk factor for its occurrence. Of note, the immune response against the pathogen seems to be a determining factor for gastric oncogenesis, and increasing evidence have emphasized several host and bacterium factors that probably influence in this setting. The development of an inflammatory process against *H. pylori* involves a wide range of mechanisms such as the activation of pattern recognition receptors and intracellular pathways resulting in the production of proinflammatory cytokines by gastric epithelial cells. This process culminates in the establishment of distinct immune response profiles that result from the cytokine-induced differentiation of T naïve cells into specific T helper cells. Cytokines released from each type of T helper cell orchestrate the immune system and interfere in the development of gastric cancer in idiosyncratic ways. Moreover, variants in genes such as single nucleotide polymorphisms have been associated with variable predispositions for the occurrence of gastric malignancy because they influence both the intensity of gene expression and the affinity of the resultant molecule with its receptor. In addition, various repercussions related to some *H. pylori* virulence factors seem to substantially influence the host immune response against the infection, and many of them have been associated with gastric tumorigenesis.

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Manuscript source: Invited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: Brazil

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): E

Received: February 23, 2021
Peer-review started: February 23, 2021
First decision: April 6, 2021
Revised: April 24, 2021
Accepted: June 15, 2021
Article in press: June 15, 2021
Published online: June 28, 2021

P-Reviewer: Katayama Y, Mi Y
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Wang LL



Key Words: Gastric cancer; *Helicobacter pylori*; Immune response; Virulence factors; Polymorphisms

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Core Tip: Gastric cancer affects more than 1 million people yearly, and *Helicobacter pylori (H. pylori)* infection is the main risk factor for that malignancy. Moreover, the immune response against the infection seems to play a pivotal role in gastric carcinogenesis. This article provides a broad and updated overview on the main aspects regarding *H. pylori* infection, immune response, and gastric cancer development.

Citation: de Brito BB, Lemos FFB, Carneiro CDM, Viana AS, Barreto NMPV, Assis GAS, Braga BDC, Santos MLC, Silva FAFD, Marques HS, Silva NOE, de Melo FF. Immune response to *Helicobacter pylori* infection and gastric cancer development. *World J Meta-Anal* 2021; 9(3): 257-276
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/257.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.257

INTRODUCTION

About 1 million people are diagnosed with gastric cancer and more than 700000 individuals die from this neoplasm every year[1]. That incidence makes gastric adenocarcinoma the fifth most common malignancy and the third cause of cancer-related death worldwide[2]. Among the multiple factors that influence the development of this disease, *Helicobacter pylori* (*H. pylori*) infection stands out. Gastric colonization by this gram-negative, spiral-shaped microorganism is the main risk factor for the occurrence of gastric adenocarcinoma, and worryingly it infects more than half of the world population[3]. In that context, studies have emphasized the critical role of the interplays between *H. pylori* and host immune system in carcinogenesis[4].

The immune response activation by *H. pylori* infection in the gastric mucosa occurs mainly through the triggering of pattern recognition receptors (PRRs), which leads to the activation of intracellular cascades that culminate in the secretion of proinflammatory cytokines[5]. The events taking place in the initial phase of infection lead to the recruitment of T cells, and the establishment of specific immune response profiles by T helper (Th) cells is determinant for the development of *H. pylori*-related gastric disorders[6]. *H. pylori* has several virulence factors that favor its perpetuation in the gastric hostile environment. Some mechanisms triggered by these bacterial products play pivotal roles in the regulation of the host immune response and seem to influence the genesis of gastric neoplasms[7]. On the other hand, specific host polymorphisms in genes that encode cytokines also interfere in the risk of developing the disease by altering the expression pattern of these mediators as well as the intensity of the signals that they activate[8].

Given the background, this article aims to provide a broad and updated review on how the immune response against *H. pylori* infection influences in the development of gastric adenocarcinoma, discussing the main bacterial and host variables that interfere in the pathophysiology of the disease.

IMMUNE RESPONSE ACTIVATION BY *H. PYLORI* INFECTION

Complex host immune responses involving innate and adaptive mechanisms are induced by *H. pylori* infection[9-11]. Gastric epithelium plays a pivotal role in the innate immune response to the bacterium because its cells make up the only cell phenotype in direct contact with the pathogen in conditions in which tissue damage is absent yet[12,13]. The initial contact of the gastric epithelial cells with the pathogen activates pathogen-associated molecular pattern receptors including NOD1 and toll-like receptors (TLRs). These innate host defense mechanisms trigger cell signaling

pathways that induce the activation of nuclear factor kappa B (NF-κB), activating protein-1, and interferon regulatory factors[14]. Concerning the TLRs, it has been reported that gastric epithelial cells express TLR1, TLR2, TLR4, TLR5, TLR9, and TLR10, which interact with various *H. pylori* antigens such as lipoteichoic acid, lipoproteins, lipopolysaccharide, flagellin, HSP-60, neutrophil-activating protein A, DNA, and RNA[5,15,16]. These receptors are very important to the induction of the expression of proinflammatory and antibacterial factors[5]. For example, the translocation of NF-κB to the nucleus, aiming at activating the expression of genes associated with the inflammatory process, is directly associated with the engagement of TLRs, particularly TLR2, in a myeloid differentiation primary response 88-dependent process [17]. Myeloid differentiation primary response 88 is a key TLR adapter protein used by all TLRs, except TLR3, and transmits signals that result in the induction of inflammatory cytokines[5]. However, although TLRs are the most studied receptors, *H. pylori* promotes the activation of PRRs other than TLRs. For instance, *H. pylori* peptidoglycan, delivered into host cells by the type IV secretion system or through outer membrane vesicles secreted from the bacterium, is recognized by NOD1[18-21]. As a result, the interaction between *H. pylori* and PRRs leads to the expression of inflammatory cytokines, antimicrobial peptides, and type 1 interferon (IFN) by gastric epithelial cells[14]. Subsequently, these cytokines and inflammatory mediators stimulate the recruitment of both polymorphonuclear and mononuclear cells into the gastric mucosa[22,23]. Lastly, it is important to mention that there are other cell components that also act in the induction of this inflammatory process. Recent reports have led to the conclusion that micro RNAs act as modulators of *H. pylori* infection and concomitantly have their expression affected by the bacterium[24].

This inflammatory response is characterized by the chemotaxis of monocytes/macrophages, dendritic cells (DCs), B and T cells, and in particular, neutrophils, whose main chemoattractant is the interleukin (IL)-8 secreted by gastric epithelial cells as a result of the engagement of NOD1, for instance[25,26]. Neutrophils are recruited to the lamina propria at the beginning of *H. pylori* infection, and several specific *H. pylori* factors are known to interact with these cells and modulate their responses[27,28]. One of these factors is a protein produced by *H. pylori* known as neutrophil-activating protein (HP-NAP or neutrophil-activating protein A). HP-NAP can promote chemotaxis, endothelial adhesion, and production of reactive oxygen intermediates by neutrophils[29-31]. Incubation of these cells with HP-NAP results in significant production of cytokines such as IL-12 and IL-23. The same effects of HP-NAP on cytokine secretion were also observed in macrophages and DCs. Therefore, it is possible to conclude that this protein acts on both neutrophils and monocytes, inducing the production of cytokines[30].

Mononuclear infiltration in the lamina propria is also characteristic of *H. pylori*-induced chronic infection[13]. Human monocytes and macrophages are important coordinators of the immune response to *H. pylori*-derived products and signals from epithelial cells in direct contact with the bacterium on the surface of the mucosa[15]. In this infection, both monocytes and macrophages, alongside the DCs, act as activators of adaptive immunity, because they are antigen-presenting cells, capable of expressing class II MHC molecules that activate CD4+ T cells[32]. Furthermore, monocytes and macrophages also produce factors such as IL-12, responsible for inducing a polarized Th1 immune response, IL-1β, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α), which, except for IL-10, induce the amplification of the inflammatory response[16]. Moreover, it is important to note that macrophages are also effector cells that are able to produce nitric oxide derived from the enzyme-inducible nitric oxide synthase (iNOS, NOS2) and reactive oxygen species, both associated with cellular damage[33].

Although in smaller numbers, DCs are also important in the immune response to *H. pylori* infection, especially because they represent an important bridge between the innate and adaptive immunities[26]. These cells express a broad spectrum of PRRs, which enables them to capture antigens at the periphery and induce T naive cells to direct T cell differentiation[34]. This role is played through three main signals: (1) presentation of foreign antigens in the form of peptides bound to class II MHC molecules to T cells; (2) costimulation of T cell differentiation; and (3) secretion of cytokines, particularly IL-6, IL-8, IL-10, IL-12, IL-1β, and TNF-α[35]. Both aforementioned antigen-presenting cells exhibit remarkable secretion of IL-12, which enables the induction of a Th1-polarized immune response, responsible for the secretion of INF-γ and low amounts of cytokines characteristic of Th2 responses, such as IL-4 and IL-5[36-40].

Finally, mast cells represent an additional innate cell phenotype that is found within the *H. pylori*-infected gastric mucosa. These cells can be activated by various *H. pylori* components. For instance, the bacterial virulence factor VacA can induce mast cells to

express multiple inflammatory cytokines, including IL-1, TNF, IL-6, IL-23, and IL-10 [41,42]. Upon the stimulation of epithelial cells, macrophages, and DCs by *H. pylori* bacterial factors, CD4+ and CD8+ T cells are recruited to the gastric mucosa, with preferential activation of CD4+ T cells in detriment to CD8+ cells[43-46].

IMMUNE RESPONSE PROFILES IN *H. PYLORI* INFECTION AND GASTRIC CANCER

As aforementioned, the triggering of an immune response against *H. pylori* involves the activation of CD4+ and CD8+ T cells and their migration to the gastric environment[47]. Among the cytokines expressed in that context, those inducing the differentiation of naïve T cells into Th1 (*e.g.*, IL-12), Th17 [*e.g.*, transforming growth factor β (TGF-β), IL-23, and IL-6], and regulatory T cells (Treg) (*e.g.*, IL-2 and TGF-β) cells stand out[48].

The establishment of a proinflammatory Th1 response in the *H. pylori* infection is associated with the development of corpus gastritis. Depending on further host and environmental variables, the aforementioned condition can result in gastric atrophy and intestinal metaplasia, which are well-known precancerous lesions[48,49]. Of note, our group previously demonstrated that Th1 response varies according to the age among *H. pylori*-positive individuals. In that study, we observed higher gastric concentrations of Th1-related cytokines IL-2, Il-12p70, and INF-γ in adults than in children. Moreover, the levels of Th1 cytokines were directly correlated with the severity of gastric inflammation[50].

Regarding Th17 response, although other cytokines such as TGF-β and IL-6 are strongly related to this immune profile, current evidence emphasizes the pivotal role of IL-23 in its induction in the setting of *H. pylori* infection[51,52]. A study found that chronically infected IL-23(p19)$^{-/-}$ mice had reduced gastric expression of IL-17A as well as milder gastric inflammation and higher levels of *H. pylori* colonization compared to wild-type *H. pylori*-positive mice[53]. The IL-17A, in its turn, promotes the migration of polymorphonuclear leukocytes to the infection site and is an important component in the control of *H. pylori* gastric infection[54]. Previous studies using mice have shown that IL-17A$^{-/-}$ as well as IL-17RA-defficient individuals have a milder gastric neutrophil infiltration against *H. pylori* infection than wild-type mice. Interestingly, the mice lacking IL-17RA signaling had an enhanced chronic inflammation with intense infiltration of B and CD4+ T cells into the gastric mucosa[55].

Dual roles have been attributed to Th17 responses in cancer settings. On one hand, this immune profile seems to be important in the immunosurveillance against malignant cells because it stimulates the migration of leukocytes into tumors and promotes the activation of antitumor CD8+ T cells. Intratumoral Th17 cells induce the expression of CCL20, a chemokine that attracts DCs to the tumor environment, as shown in a recently published paper by Chen *et al*[56]. Subsequently, DCs phagocytose tumor material and migrate to lymph nodes, contributing to the activation of CD8+ T cells that migrate to the tumor environment through their chemotaxis to the Th17-induced CXCL9 and CXCL10[57]. Moreover, studies have shown that Th17 cells can convert into Th1 lymphocytes *in vivo*, enhancing their antitumor effectiveness[58,59]. When stimulated by IL-23 and IL-12 in an environment with absent or low TGF-β, Th17 cells are able to express IFN-γ and T-bet, important Th1-related molecules. Interestingly, Th17-derived Th1 cells have a more effective antitumor activity compared to other Th1 lymphocytes, and this may be due to the prolonged survival and superior functionality of the former compared to the latter[60].

On the other hand, the Th17 profile is involved in various protumor activities. First, IL-17 seems to promote angiogenesis because elevated intratumoral levels of that cytokine are associated with high expression of vascular endothelial growth factor and increased tumor vascular density[61]. Complementally, the aforementioned cytokine stimulates cancer cells to release IL-6, which besides promoting vascular endothelial growth factor production enhances signal transducer and activator of transcription 3 activation, increasing the survival of malignant cells by suppressing apoptosis[62]. Moreover, studies have described the existence of FOXP3^{+} CD4^{+} Th17 cells, which may play regulatory, protumor roles in cancer contexts. This phenomenon seems to occur along with low levels of Il-6 and IL-23 as well as with the presence of TGF-β, which activates FOXP3 expression[63].

A study carried out by Su *et al*[62] showed that IL-17 and RORγt (the main IL-17A transcription factor) were highly expressed in both tumor microenvironment and peripheral blood mononuclear cells (PBMCs) of gastric cancer patients, mainly among

those with metastasis. This data suggests that the presence of Th17 cells is directly associated with the occurrence of gastric cancer and with the severity of the disease. Indeed, a recently published study embracing stage IV gastric cancer patients from four cohorts have reinforced that theory because it found abnormally high levels of Th17 cell differentiation and activation of IL-17 pathways among patients with severe disease[64]. Interestingly, another study evaluating the percentages of Th17 cells in PBMCs among gastric cancer patients before and after tumor resection observed a significant drop in the proportion of Th17 cells after the treatment[65]. Of note, IL-27 has been highlighted as a crucial cytokine that plays dual roles in the regulation of the immune system. As far as this cytokine enhances T-bet expression through IL-27/IL-27Rα signaling and subsequent signal transducer and activator of transcription 1 phosphorylation leading to Th1 cell differentiation, IL-27 impairs Th17 responses by downregulating RORγT[66,67]. In a recently published study, our group showed that *H. pylori*-infected individuals have higher IL-27 levels in their serum and gastric mucosa than non-infected individuals. In contrast, there was a lack of IL-27 in both serum and gastric environment of gastric cancer patients, who also showed a remarkable Th17-polarized inflammatory pattern[68].

The Treg cells might play pivotal roles in *H. pylori*-induced gastric adenocarcinoma by favoring infection perpetuation and by repressing immune responses against malignant cells through the secretion of regulatory cytokines. Indeed, studies have observed that Treg cells are positively correlated with increased bacterial colonization [69], being also increased among gastric cancer patients[70,71]. Three types of Treg cells have been described by studies: IL-10-secreting Tr1 cells, TGF-β1-producing Tr3 cells, and FOXP3-expressing CD4+CD25high Treg cells[72]. The latter is a pivotal component in the scenario of *H. pylori* colonization, favoring the pathogen persistence in the gastric environment by suppressing the immune responses. A study demonstrated that FOXP3, TGF-β1, and IL-10 are highly expressed during *H. pylori* infection, and the density of FOXP3+ Treg cells was higher in the gastric mucosa of infected individuals than in *H. pylori*-negative people. These cells have been associated with increased bacterial density among individuals with gastritis[73].

Advances in the understanding of the interplays between Treg responses and the development of gastric cancer have been achieved. Interestingly, a recent study including gastric cancer patients in various stages of the disease showed that high infiltration of $FOXP3^+$ Treg cells was associated with poor outcomes among individuals with advanced disease, but it was a predictor of better prognosis among patients with early-phase disease[74]. Current evidence emphasizes the role of Wnt/β-catenin signaling in gastric carcinogenesis because 70% of gastric cancer patients have dysregulation in pathways associated with this signaling[75]. β-catenin induces gastric cancer cells to produce CCL28, which strongly attracts Treg cells to the tumor environment. In this sense, a recently published study using *Helicobacter felis*-colonized mice with gastric cancer found the block of β-catenin-induced CCL28 through anti-CCL28 antibodies leads to the suppression of gastric cancer progression by inhibiting Treg cells infiltration[76].

A surface glycoprotein known as neuropilin-1 seems to be crucial for the immunoregulatory events taking place in the tumor environment. The role of that molecule had already been well described in other malignancies, being related to cell migration, angiogenesis, and invasion[77]. In a new investigation by Kang *et al*[78], the expression of neuropilin-1 was associated with increased levels of the regulatory cytokines IL-35, IL-10, and TGF-β1 as well as with increased infiltration of Treg cells and M2 macrophages in gastric cancer. Moreover, its expression was positively correlated to poorer outcomes, which indicates that neuropilin-1 has the potential to be used as a prognostic factor in gastric cancer patients.

Besides the aforementioned roles of Treg cells in gastric cancer development, Liu *et al*[79] found that these cells promote the expression of leucine-rich repeat containing G protein-coupled receptor 5 by tumor cells *via* TGF-β1 and TGF-β1 signaling pathway, probably involving the aforementioned Wnt/β-catenin signaling. The leucine-rich repeat containing G protein-coupled receptor 5 is a global stem cell marker whose overexpression is observed in gastric cancer, and it has also been positively correlated with tumor invasion, metastasis, and poor prognosis among individuals with that malignancy[80]. Figure 1 summarizes the roles played by Th cells in the setting of gastric carcinogenesis.

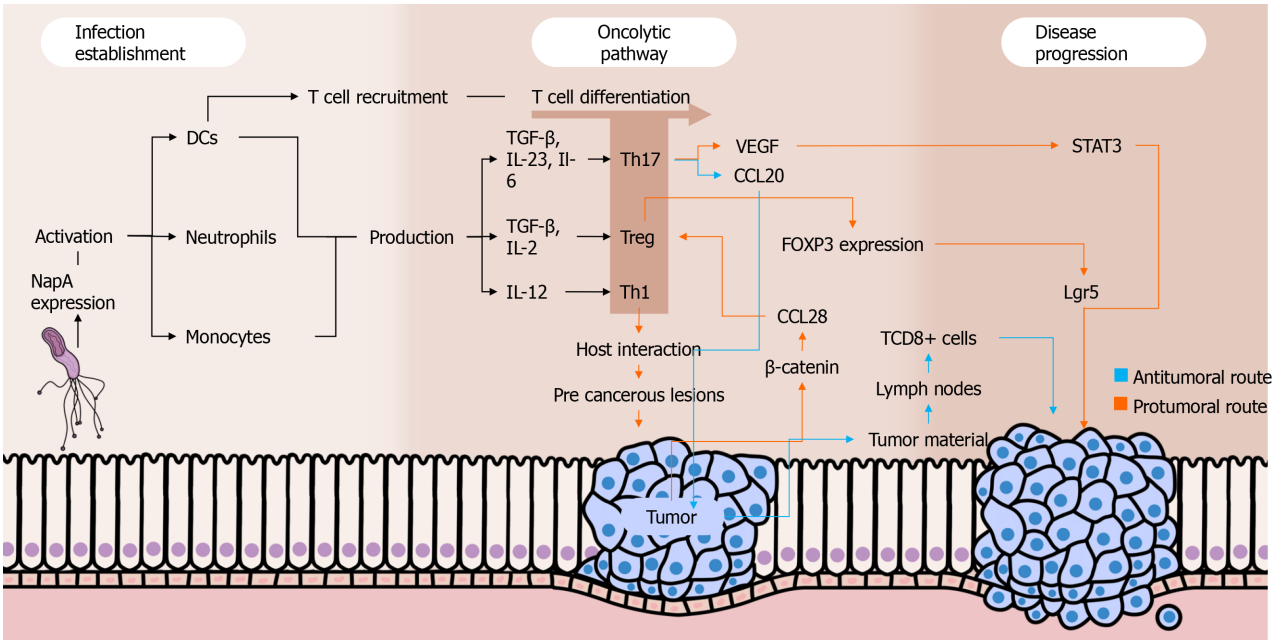


Figure 1 Interplays between *Helicobacter pylori*, immune response, and gastric cancer. DCs: Dendritic cells; IL: Interleukin; Lgr5: Protein-coupled receptor 5; NapA: Neutrophil-activating protein A; STAT3: Signal transducer and activator of transcription 3; TGF-β: Transforming growth factor β; Th: T helper; VEGF: Vascular endothelial growth factor.

POLYMORPHISMS IN GENES THAT ENCODE CYTOKINES AND GASTRIC CANCER

IL-1

The interleukin-1 family has 11 molecules that are able to interact with almost all human cells[81]. Among these, the proinflammatory IL-1β and the antagonist receptor of IL-1 (IL-Ra) have been associated with an increased risk of developing gastric cancer [82-85]. The genes encoding IL-1β and IL-1Ra are called *IL1B* and *IL1RN*, respectively [86]. Single nucleotide polymorphisms (SNPs) found in these genes alter the inflammatory response of these cytokines. The SNPs identified in the coding of IL-1B have a C-T transition base at -511, -31, or +3954 positions. For IL-Ra, the allele 2 (IL-RN*2) has been associated with inflammatory responses that increase the risk of disease development[87].

In the presence of any of the three aforementioned SNPs in the sequences that encode IL-1β, the production of this cytokine can be enhanced. This overexpression has been associated with the development of hypochlorhydria and atrophy of the gastric corpus, in addition to an increased risk for gastric cancer, especially on *H. pylori* positive subjects[88-98]. The production of IL1-Ra is mediated by a diversity of cytokines such as IL-1β, and the antagonistic function of the former controls the inflammatory response of the later. In this sense, the SNP IL-1RN*2 has been linked to an increased secretion of IL-1β[99-102].

IL-8

IL-8, also known as CXCL8, is a proinflammatory chemokine from the alpha subfamily (CXC)[103]. It can be produced by several cells such as epithelial and endothelial cells, monocytes, macrophages, and tumor cells[104,105]. Increased expression of IL-8 is promoted by various stimuli, including the initiation, modulation, and maintenance of the host inflammatory response against *H. pylori* infection[106,107]. This molecule induces the migration and proliferation of endothelial cells, contributing to angiogenesis and tumorigenesis, being related to increased cell migration, invasion, and metastasis[108].

The *CXCL8* gene is located in chromosome 4q12-21 and possesses three introns, four exons, and a proximal promoter region[109]. The genetic polymorphism IL8-251T> A (rs4073) has been associated with variations in the expression of IL-8 and increased risk of gastric cancer development mainly in Brazilian, Chinese, and Korean populations[110]. Curiously, that polymorphism was not significantly associated with a higher risk of gastric cancer in the Japanese population, which might be related to

specific environmental factors and genetic background[103].

IL-10

As previously discussed in this review, IL-10 is an anti-inflammatory cytokine; therefore, it inhibits the activity of some defense cells and limits the production of proinflammatory cytokines[111]. Polymorphisms -1082, -592 and, less frequently, -819, can modulate IL-10 transcription, decreasing the expression of this cytokine, which can initiate a hyperinflammatory response that increases the risk of gastric lesions and cance[112-116]. Therefore, these polymorphisms have been associated with a possible higher risk of gastric cancer, mainly in Asian populations but also in a study conducted with American subjects[117-120].

IL-2

IL-2 is a cytokine that plays proinflammatory and anti-inflammatory roles and is encoded by a gene located in chromosome 4q21. Among other repercussions, this molecule contributes to the proliferation of T regulatory cells and regulates the expansion and apoptosis of activated T cells[25,39]. The *IL2* GG variant genotype –330T> G in *H. pylori*-positive Asians and Brazilians as well as the SNP IL-2 + 114T> G and 330g/+ 114T haplotype in *H. pylori*-positive Brazilians have been associated with an increased risk of developing gastric cancer[121].

IL-4

Similar to IL-2, IL-4 also plays dual roles in the immune system, being encoded by a gene in chromosome 5q31.1[108]. Its function in tumor progression is mainly related to the inhibition of proinflammatory cytokines in the setting of antitumor immune responses, favoring the perpetuation of malignant cells[107]. Polymorphisms in *IL4* -590C/T rs2243250 CC, genotype CT + CC, and *IL4* haplotypes have been found to be associated with a higher risk of developing gastric cancer in the Chinese population [122].

IL-6

IL-6 is a cytokine that plays roles as an proinflammatory immune mediator and as an endocrine regulator[79]. This protein is encoded by a gene located in chromosome 7 and has been found to be increased in *H. pylori*-positive individuals[108]. Polymorphisms in the *IL6*-174C allele and *IL6*-174CC genotype are associated with an enhanced prevalence of diffuse-type gastric cancer, whereas the *IL6*-174CG has been related to intestinal-type gastric cancer[106]. In addition, the *IL6* SNP -572 (G> C, rs1800796) has been emphasized as a potential genetic biomarker for increased gastric cancer risk in Asian populations[110].

IL- 22

IL-22 is an anti-inflammatory cytokine that belongs to the IL-10 family. It participates in mucosal repair and epithelial immunity processes[123]. Chinese individuals with the SNP rs1179251 (allele G) encoding IL-22 showed a higher risk of developing gastric cancer associated with *H. pylori*[124]. Some SNPs of this cytokine have also been found in Chinese patients with increased risk for MALT gastric lymphoma induced by *H. pylori* (alleles C in rs2227485; A in rs4913428; A in rs1026788 and T in rs7314777)[125].

H. PYLORI VIRULENCE FACTORS, IMMUNE RESPONSE, AND GASTRIC CARCINOGENESIS

CagA

Infection with *cag*A-positive *H. pylori* strains is the main risk factor for the development of gastric cancer[126-129]. CagA is a multifunctional, pore-forming protein that induces vacuolization, cell necrosis, and cell apoptosis in gastric epithelial cells[130-134]. Of note, this virulence factor appears to induce an important modulation of the host immune system[135,136]. A recently published study by He *et al*[137] using mice revealed that CagA suppresses the expression of proinflammatory cytokines induced by *H. pylori* infection through the inhibition of the mitogen-activated protein kinase and NF-κB pathways. In addition, the study has shown, for the first time, that this virulence factor downregulates the posttranslational modification of TRAF6, obstructing the transmission of a signal downstream responsible for promoting the release of proinflammatory mediators.

Studies have described that *H. pylori* has a molecular mechanism of *Cag*A expansion through which its number of copies expands, consequently enhancing its virulence [138,139]. The analysis of the PMSS1 *H. pylori* strain showed that bacteria that carry more *Cag*A copies also produce more toxin, leading to enhanced cell elongation and IL-8 induction[140,141]. Yamaoka *et al*[142]. proposes that the levels of IL-8 of the gastric mucosa are related to the presence of CagA and OipA. Both molecules seem to be involved in the induction of interferon regulatory factors and play a role in the complete activation of the IL-8 promoter, using different convergence pathways[143].

VacA

Vacuolating cytotoxin A (VacA) is a protein encoded by a monocistronic gene known as *vacA*. Secreted VacA molecules are 140 kDa initially, but they are rapidly cleaved into a 10 kDa domain (p10) to produce a mature 88 kDa protein[144,145]. Generally, they are secreted as soluble proteins in the extracellular space; however, they are found on the bacterial surface as well[146]. Moreover, this virulence factor is expressed by almost all *H. pylori* strains[147].

VacA inhibits activation and proliferation of T and B cells, a process that induces the apoptosis of macrophages mainly through the inhibition of INF-β signaling. Moreover, this virulence factor induces an excessive release of IL-8[148]. Specifically in T cells, VacA inhibits the production of IL-2, in addition to regulating the surface expression of the IL2-α receptor. This process is possibly due to the ability of VacA to inhibit the activation of the nuclear factor of activated T-cells, a global transcription factor that regulates immune response genes for T cell activation. The mechanism by which VacA inhibits activation of nuclear factor of activated T-cells is uncertain; however, it is believed that this virulence factor influences the calcium flow in the extracellular medium, which inhibits the calcineurin-dependent Ca^{2+}-calmodulin complex[149]. Other effects on these cells include the activation of intracellular signaling through MAP kinases, such as MKK3/6 and p38 as well as the Rac/Vav-specific nucleotide exchange factor[145]. Studies with primary CD4+ T cells in humans have demonstrated that VacA inhibits the proliferation of activated T cells through a mechanism that is independent of the effect of VacA on nuclear factor of activated T-cells activation and IL-2 expression[150,151]. In antigen presenting cells, VacA seems to interfere with the formation of vesicular compartments in macrophages infected with *H. pylori* causing homotypic vacuolar fusion and consequent changes in their physiological properties. It has also been reported that VacA can interfere with the antigen presentation of B lymphocytes by interfering in the MHC II of these cells. Finally, blocking the activation and proliferation of this set of cells helps *H. pylori* to resist the host immune response, establishing a persistent infection and with worse clinical outcomes[146].

The various positive VacA-linked bacterial genotypes are associated with a higher prevalence of malignant gastric lesions, in addition to a greater severity of inflammation induction by the pathogen. VacA is the most studied toxin in *H. pylori* due to its versatility in relation to different receptors in different cell types and functions. VacA is directly involved in the formation of intracellular vacuoles, which provide the survival of the bacteria in the gastric environment, even after drug treatment. Therefore, other studies need to be developed with VacA in order to better understand the persistence of the pathogen in the gastric environment[152].

Duodenal ulcer promoter A

The duodenal ulcer promoter A (DupA) protein is an *H. pylori* virulence factor whose gene is located in the plasticity zone of the bacterial genome[153]. The *DupA* gene contains two overlapping open reading frames (jhp0917 and jhp0918) that form a continuous locus[154]. Of note, only strains that harbor both aforementioned segments are able to produce the DupA protein[155].

The initial studies on DupA show that its pathogenicity is closely linked to the development of duodenal ulcer[154]. Based on *in vitro* and *in vivo* studies, such an outcome is believed to be due to the role of the *DupA* gene in the activation of NF-κB and activating protein-1, which enhance the infiltration of neutrophils with consequent expression of IL-8 in the antrum that promotes risk of these injuries[156,157]. These findings have shown that predominant antral gastritis often leads to a reduction in somastatin, greater gastrin secretion, and consequently greater release of gastric acid and formation of duodenal ulcer[158]. In this context, DupA expression is negatively correlated with the risk of gastric atrophy, intestinal metaplasia, and gastric cancer [159,160]. However, it has to be emphasized that DupA has not been associated with the development of duodenal ulcers in western populations[161,162].

OipA

The 34 kDa external inflammatory protein A (OipA), encoded by the *hopH* gene (hp0638), located approximately 100kb from the Cag Pathogenicity Island, belongs to the family of External Membrane Proteins. This protein is associated with gastric inflammation, being one of the main *H. pylori* virulence factors[163].

The attachment of gastric epithelial cells through OipA occurs with the induction of cellular apoptosis *via* the Bcl-2 pathway, increased levels of Bax, and cleaved caspase 3 [164]. Notably, several studies have shown that positive OipA has been more frequent in individuals with precancerous lesions than those with gastritis alone[149,165-167].

The *OipA*-positive *H. pylori* strains are more prone to gastric colonization, being also associated with a higher risk of peptic ulcer disease and gastric cancer. This molecule strongly induces inflammation, and the infiltration of neutrophils as well as the production of IL-8 are significantly higher in o*ipA*-positive strains compared to the negative ones[168]. Some studies indicate that OipA induces the interferon regulatory factors 1, which binds and activates the element similar to the response element stimulated by IFN, to induce the genetic transcription of IL-8 and its production[153]. In addition, NF-κB and activating protein-1 are also involved in the genetic transcription and production of IL-8 by gastric epithelial cells infected with *H. pylori* [169].

Other proinflammatory cytokines may also be present in *H. pylori* infection caused by the presence of OipA, such as IL-1, IL-6, IL-8, IL-11 IL-17, matrix metalloproteinase-1, TNF-α, or CC chemokine ligand 5[170]. This is similar to a response linked to the Cag Pathogenicity Island[8]. However, depending on the OipA states in different strains of *H. pylori*, the secretion of these cytokines may not be observed[171-173].

The genes that express functional OipA are strong factors of bacterial virulence and are linked to the genotypes VacA s1, VacA m1, blood group antigen-binding adhesin 2, and the Cag Pathogenicity Island gene and can act synergistically with each other to induce worse clinical outcomes of diseases caused by *H. pylori*[155].

IceA

The gene induced by contact with epithelium A (*iceA*) is a virulence marker still poorly described. The functions related to this virulence factor remain unclear and it possesses two variants: IceA1 and IceA2. *H. pylori* has only one *iceA* locus from which the protein can be expressed. Therefore, the presence of both aforementioned variations indicates an infection by different strains of the pathogen[149].

The IceA relationship and the clinical outcomes of gastric diseases are still controversial[174]. However, studies have emphasized that strains positive for this gene induce the release of the proinflammatory cytokines IL-6 and IL-8 more intensely than negative strains[175,176]. Feliciano *et al*[177] demonstrated the possible role of IceA1 in the development of gastric cancer but not in peptic ulcers. In addition, Yakoob *et al* [178] demonstrated that *iceA2*-positive *H. pylori* strains were more often associated with chronic active inflammation, gastric ulcer, and gastric adenocarcinoma. Studies indicate that IceA has its function preserved regardless of the presence of other *H. pylori* virulence factors[179-181].

To date, there seems to be a consensus that the global prevalence of IceA1 is higher than IceA2[174]. However, although there is a greater expression of IceA1 than IceA2, the latter is associated with greater granulocytic and lymphocytic infiltration as well as atrophic gastritis[182]. Abu-Taleb *et al*[183] demonstrated in their study that *H. pylori*-infected individuals who express IceA1 or IceA2 alone do not develop gastric carcinoma. On the other hand, 75% of the patients who had both alleles (IceA1/IceA2) concomitantly developed gastric carcinoma. Strains that have positive IceA2 tend to stimulate IL-1, resulting in an increased risk of precancerous lesions in the gastric mucosa. This process can become worse if associated with the concomitant effects of other virulent factors, worsening inflammatory processes. Taken all together, the *iceA* gene is an important marker of severe gastric diseases that must be taken into account [149].

BabA

The mechanisms related to the BabA pathogenicity are still poorly elucidated. Nonetheless, studies have shown that BabA-dependent *H. pylori* cell adhesion have great relevance in the initial colonization of the pathogen[184]. Moreover, BabA works by facilitating the entry of CagA and VacA into host cells[185]. BabA-negative *H. pylori* strains have been associated with the development of mild gastric lesions and are rarely associated with gastric cancer. This means that BabA positivity might increase the risk of serious gastric lesions and carcinomas[186].

Table 1 Roles of *Helicobacter pylori* virulence factors on host immune response

Virulence factor	Effects over immune response	Ref.
CagA	IL-8 stimulation; Suppression of proinflammatory cytokines through the inhibition of MAPK and NF-κB	Schumacher and Schwarz[81] and Garlanda *et al*[83]
VacA	Inhibition of activation and proliferation of T and B cells; Induction of the apoptosis of macrophages (inhibition of INF-β signaling); IL-8 stimulation; Inhibition of IL-2 production by inhibiting NFAT; Impairment of antigen presentation by B lymphocytes	Drici *et al*[86] Dinarello[89], Hollegaard and Bidwell[90], Sicinschi *et al*[91] and Garza-González *et al*[92]
DupA	Promotion of neutrophilic infiltration	Zeng *et al*[94]
OipA	Promotion of neutrophilic infiltration; Stimulation of IL-1, IL-6, IL-8, IL-11, IL-17, MMP-1, TNF-α, and IRF-1	Gehmert *et al*[97]
IceA	Stimulation of IL-1, IL-6, and IL-8	Abbasian *et al*[101]
BabA	Stimulation of IL-33	Xue *et al*[108]

BabA: Blood group antigen-binding adhesin; DupA: Duodenal ulcer promoter A; IL: Interleukin; INF-β: Interferon-beta; IRF-1: Interferon regulatory factor 1; MAPK: Mitogen-activated protein kinase; MMP-1: Matrix metalloproteinase-1; NF-κB: Nuclear factor-κB; NFAT: Nuclear factor of activated T-cells; OipA: External inflammatory protein A; TNF: Tumor necrosis factor; VacA: Vacuolating cytotoxin A.

A study showed a greater expression of *IL-33* mRNA in biopsies from patients infected with *H. pylori* compared to noninfected individuals. Interestingly, a direct relationship was observed between blood group antigen-binding adhesin 2 and increased gastric levels of that cytokine[187]. IL-33 plays an important role in immune regulation, providing protection after damage to epithelial cells[188]. It also has the potential to reduce colonization in gastrointestinal infections[189]. In addition, recent studies emphasize its likely role in the development of tumorigenesis[190].

Sialic acid A adhesin

Sialic acid A adhesin (SabA) is an *H. pylori* membrane protein whose expression has been explored as a biomarker for increased risk of developing gastric cancer[191,192]. Yamaoka *et al*[193] demonstrated that SabA is positively associated with gastric cancer, intestinal metaplasia, and body atrophy and is negatively associated with duodenal ulcer. *H. pylori* uses SabA to recognize the Lewis X antigen from gastric epithelial cells, and this virulence factor has been associated with non-opsonic activation of human neutrophils[194,195]. SabA mediates antigen binding to sialyl-Lewis, which is an established tumor and gastric dysplasia marker[196]. The available data on this issue highlights how harmful such adhesion can be to the gastric epithelium; however, further studies are needed to better understand the underlying immune system responses related to this molecule[197]. Table 1 shows how the *H. pylori* virulence factors interact with the immune system.

Heat-shock protein 60

Heat-shock protein 60 (hsp60) is known to have substantial immunogenic properties. Studies have demonstrated that hsp60 promotes cell signaling upon myeloid and vascular endothelial cells[198]. *H. pylori*-expressed hsp60 seems to play a role in bacterial adhesion to gastric epithelial cells and mucin[199]. In addition, that virulence factor has been shown to effectively inhibit human PBMCs. A study by Maguire *et al* [200] demonstrated that the inhibitor effect over human PBMCs was more potent with hsp60 from *H. pylori* than hsp60 from *Chlamydia pneumoniae* or human mitochondria. Evidence has shown that hsp60 also promotes immune system responses through the activation of TLRs in human gastric epithelial cells and induces IL-8 expression through TLR-2 and mitogen-activated protein kinase pathways in human monocytes [201,202]. Another study evaluating the effects of hsp60 over human monocytes demonstrated that it seems to promote an upregulation of cytokines such as IL-1a, IL-8, IL-10, IFN-γ, TNF-α, and TGF-β[203]. Regarding the oncogenic roles related to this molecule, an enhanced gastric cancer cell and promotion of tube formation by umbilical vein endothelial cells have been positively associated with hsp60, but effects on cell proliferation and cell death prevention have not been attributed to the protein [204].

CONCLUSION

The knowledge on the relationship between *H. pylori* infection, the immune system, and oncogenesis is crucial for the understanding of the mechanisms involved in the onset and progression of gastric cancer. Although considerable advances have been achieved in this research field, much has to be done in order to describe underlying mechanisms related to the *H. pylori*-related carcinogenesis. A better comprehension on this issue could be useful for the development of tools that may aid in the prevention as well as in the prognostic prediction and treatment of such an important disease. Here, we gathered data showing that *H. pylori* infection promotes multiple immune response activities, such as Th cell polarizations that are closely related to mechanisms associated with gastric carcinogenesis.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 June 28; 9(3): 277-285

DOI: 10.13105/wjma.v9.i3.277

ISSN 2308-3840 (online)

MINIREVIEWS

Nusinersen, an exon 7 inclusion drug for spinal muscular atrophy: A minireview

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Author contributions: Behera B performed the literature review and wrote the manuscript.

Conflict-of-interest statement: The author has no conflicts of interest and has nothing to disclose.

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Manuscript source: Unsolicited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: India

Peer-review report's scientific quality classification

Grade A (Excellent): 0

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Abstract

Spinal muscular atrophy is an autosomal recessive neuromuscular disease with incidence of 1 in 5000 to 10000 live births and is produced by homozygous deletion of exons 7 and 8 in the *SMN1* gene. The *SMN1* and *SMN2* genes encode the survival motor neuron protein, a crucial protein for the preservation of motor neurons. Use of the newer drug, Nusinersen, from early infancy has shown improvement in clinical outcomes of spinal muscular atrophy patients.

Key Words: CHERISH; Nusinersen; Spinal muscular atrophy; Survival motor neuron; NURTURE

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Core Tip: Spinal muscular atrophy is an autosomal recessive neuromuscular disease, with incidence of 1 in 5000 to 1 in 10000 live births. This review provides an elaborative knowledge regarding the current most effective drug for spinal muscular atrophy, Nusinersen. A brief discussion on other treatment modalities that are under trials is also provided.

Citation: Behera B. Nusinersen, an exon 7 inclusion drug for spinal muscular atrophy: A minireview. *World J Meta-Anal* 2021; 9(3): 277-285
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/277.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.277

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease, with incidence of 1 in 5000 to 1 in 10000 live births[1,2]. It is caused by homozygous

Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: December 3, 2020
Peer-review started: December 3, 2020
First decision: May 6, 2021
Revised: May 20, 2021
Accepted: June 17, 2021
Article in press: June 17, 2021
Published online: June 28, 2021

P-Reviewer: Kerpel-Fronius S
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Wang LL

deletion of exons 7 and 8 in the *SMN1* gene[1,2]. It is a rare genetic disease that occurs due to degeneration of motor neurons in the spinal cord and brainstem[3]. Clinical manifestations can appear prior to birth to early adulthood and usually manifests with symmetrical, proximal muscle weakness, which is progressive and associated with muscle atrophy. SMA patients have difficulties like inadequate weight gain, restrictive lung disease, scoliosis, joint contractures, and sleep problems but do not have cognitive issues. SMA causes mutation in chromosome 5q11.2-q13.3, affecting the survival motor neuron (*SMN*) gene, which results in lack of *SMN1* exon 7. For the preservation of motor neurons, SMN protein is crucial, which is encoded by *SMN1* and *SMN2*. A homozygous deletion of *SMN1* exon 7 is diagnostic confirmation for SMA. A typical patient with SMA can have variable copies of *SMN2* but have zero copies of *SMN1*[3-6]. The severest is type 0 and has a antenatal onset, whereas the commonest type of SMA is type1[4]. SMA type 2 presents around 6 mo to 18 mo, type 3 after 18 mo, and type 4 beyond 5 years[7]. The introduction of Nusinersen, from early infancy, has shown improvement in clinical outcomes of SMA type 1 patients. Open label phase two and phase three trials have demonstrated effective improvement of life expectancy in most of the patients[8].

STRUCTURE AND MECHANISM OF ACTION

Research have shown an intronic splicing silencer N1 (ISS-N1) sequence in intron 7 of *SMN2*, which has shown involvement in messenger RNA (mRNA) exon 7 skipping. Improvement in SMN2-mRNA exon 7 inclusion has been revealed by inhibiting ISS-N1 by antisense oligonucleotides (ASOs), and thus showing clinical improvement in SMA[9]. Nusinersen is a modified 2′-O-methoxyethyl phosphorothioate ASO. After binding to *SMN2*, it alters splicing of *SMN2* pre-mRNA, which leads to rise in exon 7 incorporation into *SMN2* mRNA. This results in an augmented manufacture of standard length SMN protein, which is obligatory for the preservation of motor neurons, but is lacking in SMA patients[10,11].

PHARMADYNAMICS AND PHARMACOKINETICS

Nusinersen is unable to cross the blood-brain barrier, when given through intravenous or subcutaneous routes. It is administered intrathecally as it unambiguously targets the central nervous system[10-12]. 3′ and 5′ exonuclease-mediated hydrolysis is the process of metabolism, and it is eliminated by the kidney[10,11]. The mean plasma and central nervous system terminal elimination half-lives are 63-87 d and 35-177 d[10,11]. The plasma and the cerebrospinal fluid volume of distributions are 29 L and 0.4 L[10, 11]. Body weight is the only variable that affects Nusinersen pharmacokinetics. There is no dose-related toxicity established in studies so a fixed dose is recommended[10, 11].

USES

In an open label phase 2 study, 20 patients with ages ranging from 3 wk to 7 mo, with infantile-onset SMA symptoms and having SMN1 homozygous gene deletion or mutation, were given several intrathecal doses of Nusinersen (6 mg and 12 mg dose). In the 12 mg dose group, there was improvement in achieving motor milestones ($P < 0.0001$), enhancements in CHOP-INTEND[13] motor function scores ($P = 0.0013$) (a dependable score for motor skills in SMA), and amplified compound muscle action potential amplitude of the ulnar nerve ($P = 0.0103$) and peroneal nerve ($P < 0.0001$)[8]. None of the patients required prolonged pulmonary support nor were any deaths reported[8]. In NURTURE study, all 25 participants achieved the ability to sit without support, 23/25 (92%) achieved walking with assistance, and 22/25 (88%) achieved walking independently[14]. Another larger "ENDEAR" randomized controlled trial comprising of 122 , ≤ 7 mo genetically proven SMA patients also revealed improvement in motor milestones, assessed by section 2 of the Hammersmith Infant Neurological Examination (HINE-2), after 13 mo of trial[15,16]. In the interim analysis, the group of patients who received Nusinersen had significant enhancement in motor-milestone response [21 of 51 infants (41%) as compared to the control group 0 of 27 (0%), *P* value of < 0.001]. In the treatment group, 22% achieved full head control, 10%

were able to roll over, 8% were able to sit independently, and 1% were able to stand; in the control group, no infants achieved these milestones. The study also concluded that the group of patients treated with Nusinersen had substantial development in motor-milestone response [37 of 73 infants (51%) as compared to control group 0 of 37 (0%)]. In comparison to the control group the patients treated with Nusinersen had a higher likelihood of event-free survival with hazard ratio (HR) for death or the use of permanent assisted ventilation of 0.53 and $P = 0.005$. The Nusinersen group had a greater likelihood of overall survival (HR for death, 0.37; $P = 0.004$)[15].

CHERISH study, a phase 3 randomized controlled trial, reported an accelerated progress in the motor milestone achievements at 13 mo, in the treatment group of 126 of 179 screened children of age 2 years to 12 years, having a genetic diagnosis of SMA [17]. In another phase 1b/2a study with SMA children of 2-15 years of age, who received ascending multiple doses (3, 6, 9, 12 mg) of Nusinersen, the Hammersmith-Functional-Motor-Scale–Expanded, Upper-Limb-Module, 6-Minute-Walk-Test (6MWT), compound muscle action potential, and quantitative multipoint incremental motor unit number estimation and safety were used to assess enrolled patients. Eleven children of type II SMA and 17 of type III SMA were enrolled. Mean Hammersmith-Functional-Motor-Scale–Expanded scores, Upper-Limb-Module scores, and 6MWT distances improved by the day 1150 visit[18].

A cohort study of 33 children with SMA1, 8.3 mo to 113.1 mo of age, were treated with Nusinersen. Evaluation was done before starting of treatment (M0), after 2 mo (M2), and 6 mo (M6) of starting treatment. Data of survival, respiratory, nutrition, and motor function assessment with the modified HINE-2 were recorded. Six months after the treatment, median development on the modified HINE-2 score was 1.5 points ($P < 0.001$), and there was a significant increase in the requirement of respiratory support [19]. No serious or life threatening adverse events (AE) were noted in any trials.

Patients with SMA usually present with respiratory morbidities like hypoventilation, aspirations, repeated lung infections due to stiffened rib cage, micro-collapse of the lungs, which occurs as a result of decreased chest wall movement, and increasing thoracic scoliosis[20]. Polysomnograms have proven early type 1 respiratory failure even in stable SMA patients. Nusinersen therapy might have improvement in functioning of peripheral muscles along with improvement of respiratory muscle and diaphragmatic functions, leading to improved co-ordination of oral secretions, decrease lung aspiration, and decreased airway disease and pneumonia.

DOSE AND ADMINISTRATION

On December 23, 2016, Nusinersen was given approval as a treatment for SMA by the Food and Drug Administration (FDA). Nusinersen 6 mg dose equivalent to be diluted to a concentration 1.2 mg/mL or 12 mg dose equivalent diluted to 2.4 mg/mL with artificial cerebrospinal fluid and administered intrathecally every 14 d for three doses, followed by a fourth dose after 30 d. The FDA recommends to administer maintenance doses once every 4 mo to maintain the tissue concentration. This drug has also been approved by European Medicines Agency[21], Australia[22], New Zealand[23], and Canada[24].

Initial therapy

Individuals who meet all of the following criteria: (1) Documentation of a confirmed diagnosis of SMA by genetic testing; (2) Documentation of ≥ 2 copies of the *SMN2* gene by genetic testing; and (3) Onset of SMA-associated symptoms before 20 mo of age.

Continuation therapy

After 6 mo of starting of therapy, continue Nusinersen therapy every 6 mo in patients meeting both criteria (1) and (2): (1) Initial therapy was determined to meet the above criteria; and (2) There is substantial improvement in SMA-associated symptoms during the previous treatment period. Clinical efficacy of Nusinersen has to be evaluated every 6 mo in the treated individual.

AE

As per ENDEAR study, severe AE in Nusinersen in 56% compared to 80% in controls

and serious AE were 76% in Nusinersen compared to 95% in control group. Even serious AEs with fatal outcome was 39% in controls compared to only 16% in Nusinersen. Pyrexia, constipation, upper-respiratory infection, pneumonia, or respiratory distress or failure was seen in ≥ 20% in Nusinersen group[15]. Almost similar results were seen in CHERISH study[17].

DILEMAS OF NUSINERSEN

Efficacy of Nusinersen in treating type 0 SMA patients

Till date there are no studies on patients who are symptomatic patients since birth or within 7 d of life being treated with the drug. Neither are there any studies on patients with one copy of *SMN2* gene being given Nusinersen therapy. Further studies are essential to demonstrate the effectiveness of this drug in these patients.

Factors affecting the response of the drug

In the CHERISH study[17], the response of Nusinersen treatment varied in SMA cases. Amongst the factors identified were the time interval when the type 1 SMA patients became symptomatic and administration of first dose of drug and the age of type 2 SMA patients. There was an extensive difference in the treatment response amongst the patients, which was not explained by these factors[15].

Type 1 SMA respiratory outcomes

None of the trials reported on swallowing evolution and even the respiratory outcomes were considered as the usage of long-lasting ventilation or not and the number of hours spent ventilated. In order to have an improved evaluation of load for families involved in the treatment of a type 1 SMA child, a better documentation of time spent in the hospital is required.

No reporting of the long-term consequences of patients who received therapy before they became symptomatic

Reports from the NURTURE study[8] have shown that children who were treated before manifesting symptoms, with three copies of SMN2, have a far improved result than when they are treated after they become symptomatic[25]. The ENDEAR study and CHERISH Nusinersen trial[15,17] have not reported on the long-term outcomes, for example the number of patients attaining major milestones of walking, standing, or sitting, whether they will be free of ventilation, and the number of patients that will have severe motor disability. Even their intellectual development assessments were not reported.

Nusinersen in type 3 and type 4 SMA

Insufficient data are available in type 3 SMA patients treated with Nusinersen, and there are no reports on type 4 SMA. A study by Montes *et al*[25] revealed an unremitting result on the 6MWT in type 3 patients, with a median surge of 98 m in a 1050-d period.

Nusinersen a very costly drug

According to a study the estimated price of care of a type 1 SMA patient who is not on Nusinersen therapy was roughly €100000 per year, and type 2 was nearly €90000 per year[26]. The cost of Nusinersen is nearly €100000 per injection and a full course of six injections are required in the initial year, and three during the subsequent year[27]. This drug has the probability to improve the existence of type 1 SMA patients from 10% to 60%[8,15] and also enhance their functional level; the higher cost is an important factor in deciding the treatment. So, identifying good responders of treatment early by various outcome measures and optimizing the treatment for presymptomatic patients through newborn screening could decrease the cost burden. So the most important challenge is Nusinersen's remarkably expensive cost[28,29].

OTHER THERAPIES

Other disease modifying therapies like *SMN1* gene replacement therapy, AVXS-101 (zolgensma) in trials, when delivered intravenously to SMA type I infants had

promising outcomes in *in vivo* studies[30-32]. Although these studies have shown improved life expectancy, motor milestones, and motor functions, the number of patients were less and additional studies are essential to prove the effectiveness. Adeno-associated virus 9 vector was most efficiently able to cross the blood brain barrier, and was able to infect almost 60% of motor neurons and enhance SMN expression[28,29]. Studies have not clearly shown long term benefits with combined treatment of Zolgensma and Nusinersen[33,34].

Some other SMN2 exon 7 inclusion drug like risdiplam (RG7916), having central and peripheral tissues distributions, have proven to be a beneficial[35,36] and has been investigated by FIREFISH and SUNFISH trials[37,38]. Branaplam (LMI070) is another such drug that interacts with U1 small nuclear ribonucleoprotein and facilitates exon 7 inclusion of SMN2 transcripts and increases SMN protein levels with initial results of ongoing trials showing improvement[39,40]. Small molecular drugs like Celecoxib, a cyclooxygenase 2 inhibitor, have revealed an increase SMN in animal models[41], and an ongoing trial is recruiting patients[42]. Quinazoline (repligen or RG3039), which blocks a decapping scavenger enzyme (DcpS) and demonstrated upsurge in FL-SMN2 transcript by enhancing SMN2 promoter activity in animal studies[43,44], did not show significant results in phase trial[45,46].

Aminoglycoside antibiotics (tobramycin, geneticin, and amikacin) act as SMN protein stabilizers by masking premature stop codon mutations and increasing read-through of exon 8 and thus increasing the SMN protein[47,48] and have only shown *in vivo* efficacy[49]. In mouse models, BBrm^2 (FDA-approved azithromycin) has revealed an upsurge in SMN and enhancement in motor function[49,50]. Similar results were shown in mouse models with Bortezomib, an ubiquitin proteasome inhibitor that inhibits SMN protein degeneration[51].

A proper SMA treatment includes both SMN-dependent and SMN-independent approaches, which will provide the central and peripheral therapies[33,52].

Neuroprotective agents like olesoxime, which has neuroprotective properties in pre-clinical studies, had suggested improvement in the function and survival of neurons [53]. Subsequently, however, they did not show significant results[52,54]. Studies with riluzole and gabapentin, did not show encouraging results[42,55-57].

As muscle weakness is eminent in SMA, SMN-independent therapies lately focus on muscle. Myostatin, a growth factor, inhibits muscle growth and by stalling its signaling pathway, muscle mass can be enhanced[58]. Follistatin[59], adeno-associated virus-mediated soluble inhibitor of activin receptor type IIB (ActRIIB)[60], in SMA mouse models and BIIB 110 (ALG 801) are undergoing a phase 1a trial[61]. In SMA mouse models[62] and phase 2 trials[63], SRK-015, a human monoclonal antibody, has shown improvement in muscle mass.

Initial trials with reldesemtiv, a skeletal muscle troponin activator, has revealed improvement in muscle function[46,64-66]. Trials are ongoing with drugs acting on neuromuscular junction, like pyridostigmine (mestinon)[67,68] and 4-aminopyridine (4-AP or ampyra)[69,70].

Studies have demonstrated that stem cell therapy in SMA provide support to degenerating motor neurons and short of functional cell replacement[71-73].

CONCLUSION

Although many therapies are under trial and some have shown some clinically beneficial results, the level of evidence is very low. Nusinersen, however, has a moderate level of evidence and has been approved by the FDA. Nusinersen can be used in diagnosed type 1, 2, and 3 SMA patients. The earlier Nusinersen is started, the better is the improvement of muscle strength and better will be the improved quality and span of life. Currently this drug is available and approved by FDA, but cost will be a limiting factor.

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World J Meta-Anal 2021 June 28; 9(3): 286-296

DOI: 10.13105/wjma.v9.i3.286

ISSN 2308-3840 (online)

MINIREVIEWS

Dengue hemorrhagic fever and cardiac involvement

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Author contributions: Leowattana W wrote the paper; Leowattana T collected the data.

Conflict-of-interest statement: The authors declare having no conflicts of interest in relation to this article.

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Manuscript source: Invited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: Thailand

Peer-review report's scientific quality classification

Grade A (Excellent): 0

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Abstract

Dengue viral infection (DVI) is one of the world's most significant viral infections spreading. Most of the patients have been asymptomatic, with relatively benign clinical manifestations and outcomes. However, a small number of patients have progressed to severe dengue diseases, including hemorrhage, multi-organ impairment, and increased vascular leakage causing hypovolemic shock, which can cause cardiovascular collapse and death. Numerous lines of evidence have demonstrated that DVI could also cause cardiac dysfunction, arrhythmias, and severe myocarditis. The treatment for dengue hemorrhagic fever (DHF) patients remains symptomatic and supportive, with close monitoring of hemodynamic status. The contributory role of cardiac dysfunction in DHF patients has potentially critical implications on the management. This review will address the current knowledge of cardiac involvement in DHF patients and the management strategy to reduce the fatality outcome.

Key Words: Dengue hemorrhagic fever; Dengue viral infection; Cardiac involvement; Cardiac injury; Myocarditis; Left ventricular ejection fraction; Tachycardia; Bradycardia; Electrocardiography; Echocardiography; Severe dengue disease

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Core Tip: The majority of dengue virus infections (DVIs) are mild; only a small number of the patients develop overt complications and fatal outcomes. Dengue can also have cardiac involvement, including myocardial dysfunction, cardiac rhythm abnormality, and myocarditis which can implicate severe dengue disease and dengue shock syndrome. Fulminant dengue myocarditis has evidence of extensive cardiomyocyte

Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 4, 2021
Peer-review started: April 4, 2021
First decision: May 13, 2021
Revised: May 30, 2021
Accepted: July 7, 2021
Article in press: July 7, 2021
Published online: June 28, 2021

P-Reviewer: Li X
S-Editor: Fan JR
L-Editor: A
P-Editor: Li JH

infection and damage, with an abnormality of electrocardiography, echocardiography, and accompanied by elevated cardiac markers. Early recognition of cardiac involvement in DVI patients, intravascular fluid replacement, circulatory overload monitoring, and preparing the essential tools for critical management are crucial in dengue hemorrhagic fever patients with cardiac involvement.

Citation: Leowattana W, Leowattana T. Dengue hemorrhagic fever and cardiac involvement. *World J Meta-Anal* 2021; 9(3): 286-296
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/286.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.286

INTRODUCTION

Dengue viral infection (DVI) is a worldwide arthropod-borne disease that has rapidly spread throughout the tropics, with the highest risk zones in Asia and the Americas[1, 2]. More than 3 billion people are living in the Aedes-infested areas and at risk of DVI [3]. Asia accounts for 75% of the DVI burden, followed by Latin America and Africa. The most significant vector *Aedes aegypti* is a daytime mosquito, capable of stinging many people in a short period of time and breeding in several types of human-made containers with water inside[4].

Dengue virus (DENV) is an enveloped virus with a single positive-stranded RNA genome, belonging to the genus *Flavivirus* of the family *Flaviviridae*. There are four distinct serotypes, designated as DENV-1, DENV-2, DENV-3, and DENV-4. The viral RNA encodes three structural proteins, namely capsid (C), pre-membrane, and envelope (E), and seven non-structural (NS) proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5[5-7]. The incubation period of DVI is 3-7 d, and the symptoms start abruptly, following three sequential phases—an initial febrile phase, a critical phase, and a spontaneous recovery phase. The febrile phase's pathognomonic features include acute onset of high-grade fever with chill, severe malaise, vomiting, and constitutional symptoms. The critical phase is characterized by varying degrees of plasma leakage into the pleural and abdominal cavities, which causes intravascular volume depletion and might progress to life-threatening dengue shock syndrome (DSS)[8]. Moreover, hemorrhagic manifestations commonly occur during the critical phase. The most common causes of death include bleeding into major vital organs, massive bleeding, and shock. The pathophysiology of shock is poorly understood and has been described as intravascular fluid loss due to increased capillary permeability and bleeding. Organ impairment occurs during this phase, as a hepatic dysfunction with elevated aminotransferase enzymes, cardiac dysfunction with myocardial injury, and a broad range of rhythm disturbances[9,10]. The recovery phase is characterized by a sudden arrest of the plasma leaks, with concomitant reabsorption of extravasated plasma and fluids. Furthermore, DENV and the NS1 are detected in blood circulation during acute DVI, and high viral load or NS1 antigenemia have been demonstrated with severe dengue diseases (SDDs), particularly in secondary infection with different serotype from the primary infection[11,12].

Viral infection of the heart is relatively common in DVI and usually of small consequence. However, it can lead to substantial myocarditis and severe acute heart failure. Acute myocarditis can present in various clinical manifestations (Table 1). The most common viruses known to cause myocarditis are shown in Figure 1. DENV is one of the viral infections of the heart. Although the human immunodeficiency virus and coxsackie B virus are important causes of myocarditis, an extensive review including them is beyond the scope of this article. Cardiac involvement in DVI is a typical intricacy, especially in a large-scale hospital setting with many dengue hemorrhagic fever (DHF) patients, and may be a contributing factor in the pathogenesis of shock and may influence the clinical outcome. Clinical features of cardiac involvement from DENV can vary widely, from asymptomatic to severe cardiac failure, which has a high fatality rate[13,14].

In 1967, Bhamarapravati *et al*[15] first reported the hearts' pathological sections from 100 DHF autopsy cases. They found that 42 of 100 patients (42%) who died from DHF had focal areas of hemorrhage in the epicardium, sub-endocardium, interstitial tissues, or a combination of such. The most common finding was the separation of myocardial

Table 1 Clinical presentation of the patients with acute myocarditis

	Clinical presentation of acute myocarditis
1	No clinical manifestations
2	Abnormal ECG or X-ray
3	Unexplained sinus tachycardia
4	Atrial or ventricular premature beats
5	Atrial or ventricular tachycardia
6	Partial or complete heart block
7	Ventricular fibrillation/cardiac arrest
8	Acute cardiac failure
9	Circulatory collapse (shock)

ECG: Electrocardiogram.

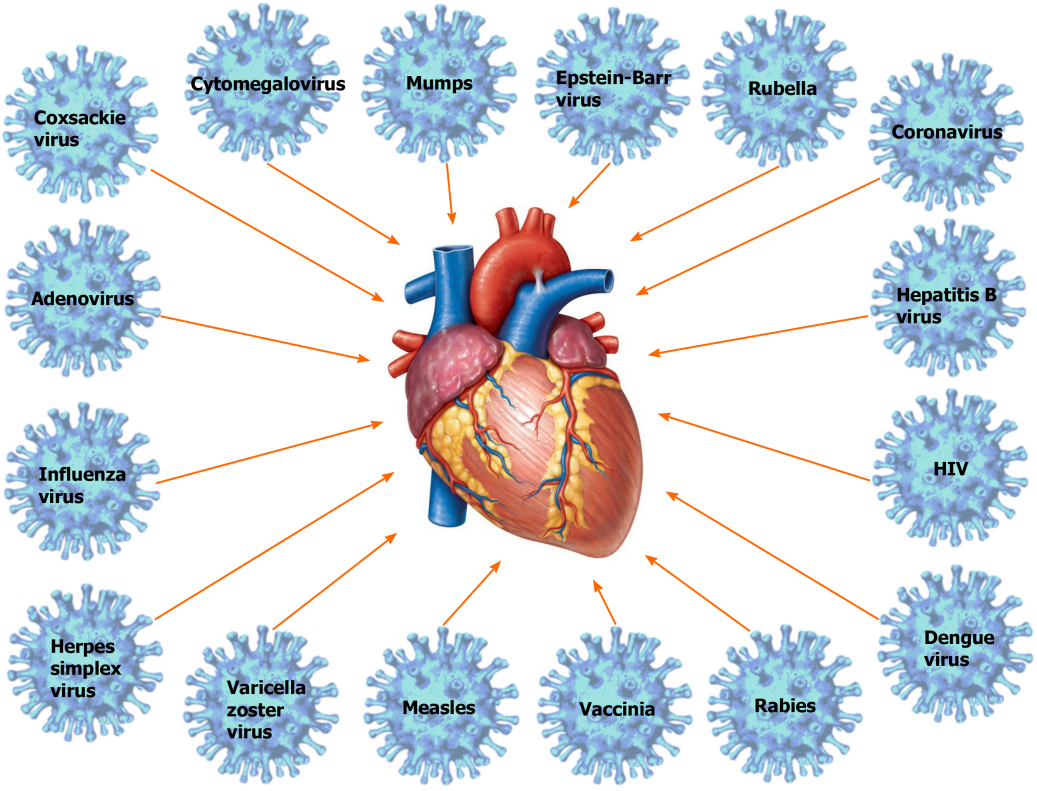


Figure 1 Viruses causing myocarditis. HIV: Human immunodeficiency virus.

fibers with interstitial tissue edema. Hemorrhage in the myocardium was extensive, with condensation and an acidophilic pattern of myocardial fibrils. There were also a few cases of interstitial infiltration by a small number of mononuclear and lymphoid cells. Moreover, several investigators have demonstrated that cardiac involvement in DVI might cause death in DHF patients[16-20]. However, clinical practice guidelines (CPGs) have only slightly addressed cardiac involvement as a mandatory concern in DHF management[21,22]. Recently, the CPG from the Pan American Health Organization included myocarditis as a crucial pathophysiologic factor in SDD[23].

This review presents the incidence, clinical manifestation, electrocardiogram (ECG) abnormalities, cardiac biomarkers, echocardiography (ECHO), and cardiac imaging in DVI patients with cardiac involvement.

INCIDENCE AND CLINICAL MANIFESTATION

In 1998, Wali *et al*[24] conducted a study to evaluate cardiac involvement in 17 consecutive patients with DHF, by use of radionuclide ventriculography, ECHO, and ECG during the epidemic of DEN-2. They found that the mean left ventricular (LV) ejection fraction (LVEF) was 41.69%, and 7 patients (41.17%) had LVEF < 40%. Moreover, global hypokinesia was detected in 12 patients (70.59%) and was found to revert to normal within 3 wk. They concluded that acute reversible cardiac involvement was present in DHF or DSS and could be responsible for clinical shock. Hence, the role of ECG, ECHO, and radionuclide ventriculography in the clinical evaluation of DHF and DSS patients should be considered tools to improve the prognosis in these patients.

In the same year, Kabra *et al*[25] conducted a study to evaluate cardiac involvement in 54 pediatric patients admitted to the hospital with various grades of DVI (DF/DHF/DSS). They found that 9/54 (16.7%) children had LVEF < 50% and 2/54 (3.70%) had LVEF < 35%. They concluded that cardiac functions need to be assessed in patients with DVI, especially those with hypotension, with adequate hydration. In 2003, Khongphatthanayothin *et al*[26] reported an observational study of LV performance and hemodynamic abnormalities during different stages of DHF. They found that LVEF and velocity of circumferential fiber shortening adjusted for end-systolic meridional wall stress and end-diastolic volume were significantly lower during the toxic stage than after recovery. Moreover, the cardiac index was found to be low during the toxic stage and to remain subnormal during the convalescence stage due to sinus bradycardia. They concluded that the mechanism of decreased cardiac output during a toxic stage in DHF patients was complex, due to decreased preload, decreased LV performance, and subnormal heart rate response (Table 2).

In 2007, Kularatne *et al*[27] investigated cardiac complications in 120 DHF patients between 13-76 years of age. They found that 75/120 (62.5%) of the patients had ECG abnormalities (*e.g.*, T inversion, ST depression, bundle branch blocks). Moreover, 5/75 (6.66%) of the patients had LVEF < 55%. They concluded that cardiac involvement in DHF was common and early recognition and differentiation from DSS is crucial. In the same year, Khongphatthanayothin *et al*[28] conducted a study to determine the prevalence of myocardial depression and its effect on the clinical severity in 91 patients with DF, DHF, and DSS. They found that during the critical stage, LVEF < 50% was present in 2 (6.7%), 5 (13.8%) and 9 (36.0%) patients with DF, DHF and DSS, respectively. Furthermore, all 14 patients in convalescent stage who had had LVEF < 50% at the critical stage presented LVEF ≥ 50%. They concluded that the proportion of patients with cardiac dysfunction was larger for those with higher clinical severity of the disease.

In 2009, Salgado *et al*[29] conducted a prospective study to determine the incidence of myocarditis in 102 DHF children. They found that 11/102 (10.7%) of the children had signs of myocarditis, 10/102 (9.8%) needed inotropic drugs, 9/102 (8.8%) had bradyarrhythmias, and 2/102 (1.9%) had tachyarrhythmias. Investigations by ECHO demonstrated pericardial leakage in 2/102 (1.96%) and diminished LVEF in 1/102 (0.98%) of the patients. They concluded that the myocarditis in DHF patients was not rare and should be of concern during DHF management. In 2011, La-Orkhun *et al*[30] evaluated the cardiac arrhythmias and heart rate variability in 35 children with DVI using overnight 18-24-h Holter monitoring. They found that 10/35 (29%) of the patients had cardiac rhythm abnormalities [sinus pause, first-degree arterioventricular (AV) block, Mobitz type I second-degree AV block (Wenckebach), atrial ectopic beats, and ventricular ectopic beats] during the convalescent stage. There was no association between the clinical severity and the incidence of cardiac arrhythmia. They concluded that various benign bradyarrhythmias and ectopic beats are present in patients with DVI during the convalescent stage.

One year later, Yacoub *et al*[18] conducted an observational study to evaluate the cardiac involvement in 79 DVI patients (age range of 8-46 years). They found that the patients with severe dengue had worse cardiac function than those with dengue, in the form of LV systolic dysfunction and with increased left myocardial performance index. Furthermore, septal myocardial systolic velocities (S') were also reduced. They concluded that the patients with severe dengue have evidence of systolic and diastolic cardiac impairments, with the septal and right ventricular wall being predominantly affected. Then, in 2013, Miranda *et al*[31] conducted a study to evaluate the cardiac involvement in 81 DVI patients. They found that 12/81 (14.81%) of the patients had elevated troponin I and NTproBNP. Of the 10 patients, 1 showed reduced LVEF, 2 showed LV segmental abnormalities with preserved ejection fraction, and 1 showed a significant pericardial effusion with tamponade; cardiac magnetic resonance

Table 2 Incidence and clinical manifestations of dengue viral infections with cardiac involvement

Ref.	Diagnosis	Study population	Incidence, %	Cardiac assessment methods	Main findings and clinical outcomes
Wali *et al*[24], 1998	DHF/DSS	17 (14-58 yr)	70.59	ECG. ECHO. Radionuclide ventriculography. Tc-99m pyrophosphate imaging	ECG showed ST and T wave changes (29.4%). Mean LVEF = 41.7%. Mean LVEF in DSS patients = 39.63%
Kabra *et al*[25], 1998	DF/DHF/DSS	54 (< 12 yr)	16.70	ECHO	LVEF < 50% in 16.7% of patients. LVEF < 35% in 3.7% of patients. Repeat ECHO after 2 mo showed improved LVEF
Khongphatthanayothin *et al*[26], 2003	DHF	24 (10.8 ± 2.8 yr)	-	ECHO (VCFC/ESS)	Lower LVEF, VCFC/ESS, CI, EDV and higher SVR during critical phase *vs* recovery phase
Kularatne *et al*[27], 2007	DF	120 (13-76 yr)	62.50	ECG. Cardiac markers	ECG abnormalities: (1) Sinus bradycardia; (2) ST-segment changes; (3) T wave changes; and (4) Right bundle branch block in 62.5% of patients; increased troponin levels in 29.4% of 17 patients
Khongphatthanayothin *et al*[28], 2007	DF/DHF/DSS	91 (5-15 yr)	17.58	ECHO. Cardiac markers	Reduced LVEF (< 50%) was found in 6.7%, 13.8%, and 36% of patients with DF, DHF, and DSS during the critical phase, respectively. No abnormal cardiac markers
Salgado *et al*[29], 2009	DF/DHF	102 (13 mo-10 yr)	10.70	ECG. ECHO	ECG: (1) Sinus bradycardia in 81.8% of 11 patients; and (2) Tachycardia in 18.2% of 11 patients, and T wave inversion in seven of 11 patients. ECHO: Pericardial effusions in 71.4% of 7 patients
La-Orkhun *et al*[30], 2011	DF/DHF/DSS	35 (5-15 yr)	29.00	ECG, 24-h Holter monitoring	During recovery phase: (1) Sinus arrhythmias; (2) First-degree AV block; (3) Mobitz type I second-degree AV block; (4) Atrial ectopic beats; and (5) Ventricular ectopic beats
Yacoub *et al*[18], 2012	DF/DHF/DSS	79 (8-46 yr)	35.00	ECG. ECHO. Cardiac markers	Abnormal ECG: (1) Sinus arrhythmias; (2) First-degree AV block; (3) T-wave changes; and (4) ST segment abnormalities. Systolic impairment in 45% of patients, diastolic impairment in 42%. Septal and right ventricular walls predominantly affected, worse in severe cases. 1 patient had elevated troponin I
Miranda *et al*[31], 2013	DF/DHF/DSS	81 (4 mo-81 yr)	14.81	Cardiac markers (troponin I and NTproBNP). ECHO. ECG. CMR	Elevated troponin I and/or NTproBNP in 14.81% of patients. 4/10 of patients had abnormal ECHO: (1) Functional abnormalities; and (2) Regional wall abnormalities. 4 patients had myocardial enhancement by CMR
Sengupta *et al*[32], 2013	DHF/Control	20 (DHF) (23 ± 8 yr); 20 (control) (23 ± 5 yr)	-	ECG. ECHO	LVEF reduced in DHF compared with controls (51.25 ± 0.96% *vs* 59.32 ± 1.26%; $P = 0.032$). DHF patients had: (1) Peak longitudinal strain in subendocardial region; (2) Circumferential strain in the subepicardial region; and (3) Radial strain
Yadav *et al*[33], 2013	DHF/DSS	67 (3 mo-14 yr)	48 (ECHO); 70 (Tei index)	ECG. ECHO. Cardiac markers	LVEF showed 48% cardiac involvement. Tei index showed 70% cardiac involvement. Tei index improved on discharge but did not normalize
Kirawittaya *et al*[34], 2015	DF/DHF/DSS	181 DF/DHF 35 Non-DF (< 15 yr)	14.50	ECG. ECHO. Cardiac markers	DHF patients with plasma leakage had: (1) Cardiac index (2695 *vs* 3188 L/min/m^2); (2) LV myocardial performance index (0.413 *vs* 0.328); (3) **S**ystemic vascular resistance (2478 *vs* 1820 dynes.s/cm^{-5}) compared to those without plasma leakage; and (4) Cardiac dysfunction was most pronounced around the time of plasma leakage
Li *et al*[35], 2016	DHF/DSS	1782 (adult)	11.28	ECG. ECHO. Cardiac markers	NSD(WS+)/SD had 46.66% myocarditis. NSD(WS-) had 9.72% myocarditis. DHF with myocarditis had longer hospitalization. Prognosis of DHF patients with and without myocarditis had no significant difference
Bhatt *et al*[16], 2020	DHF/DSS	182 (30.2 ± 12.6 yr)	7.10	ECG. ECHO. Cardiac markers	ECG abnormality in 44/182 (24%). Cardiac markers' elevation in 27/182 (14.83%). ECHO abnormality in 11/182 (6.04%)
Bhattacharjee *et al*[36], 2020	DF	84 (18-65 yr) (41 cases/43	-	ECG. ECHO. Cardiac markers	CK, CK-MB, troponin T and NT-proBNP levels in cases were higher than in controls. Troponin T was

		controls)			positively correlated with NTproBNP
Cabrera-Rego *et al*[37], 2021	DF/DHF/DSS	427 (> 18 yr)	19.70	ECG. ECHO	Rhythm disorders in 16.9%. Repolarization disorders in 3.0%. Pericarditis in 1.6%. Pericardial effusion in 0.9%. Myocarditis in 0.2%
Shah *et al*[38], 2021	DF	320 (> 18 yr)	35	ECG. ECHO. Cardiac markers	Sinus bradycardia in 19.7%. LVEF < 40% in 13.1%. Elevated troponin T in 15%. Elevated CK-MB in 10.6%. Elevated NTproBNP in 5.9%. Mortality rate was 4.38% with cardiac involvement

AV: Arterioventricular; CI: Cardiac index CK: Creatine kinase; CMR: Cardiac magnetic resonance; DF: Dengue fever; DHF: Dengue hemorrhagic fever; DSS: Dengue shock syndrome; ECHO: Echocardiography; EDV: End-diastolic volume; ECG: Electrocardiogram; LVEF: Left ventricular ejection fraction; NSD(WS)/SD: Non-severe dengue (warning sign)/severe dengue; SVR: Sustained virological response; VCFC/ESS: Velocity of circumferential fiber shortening adjusted for end-systolic meridional wall stress.

confirmed cardiac dysfunction in these 4 patients. They concluded that the heart could be affected by DENV in a considerable percentage of patients needing hospitalization. Overall, these date indicate that clinical presentations range from mild elevation of cardiac markers to myocarditis and pericarditis. These cardiac dysfunctions might be underdiagnosed in clinical practice and contribute to the high mortality rates of DHF and DSS.

Later on, Sengupta *et al*[32] conducted a prospective study that used 2D ECHO images of the LV in the four-, three- and two-chamber views and parasternal short-axis views at the basal, mid and apical levels to evaluate 40 participants (20 DHF, and 20 controls). They found that LV global ejection fraction was reduced in patients with DHF at presentation, as compared with controls. The peak longitudinal strain in patients with DHF was significantly attenuated in the subendocardial region, as compared with that in normal controls. DHF patients showed significantly higher radial strain than controls. They concluded that the identification of reduced LV longitudinal strain helps in understanding the mechanism of reduced LV myocardial performance seen in patients with DHF.

Yadav *et al*[33] conducted a study to evaluate myocardial involvement in infants and children with severe dengue using the Tei index, which combines systolic and diastolic time intervals to assess global ventricular function. They found fewer patients with severe dengue had myocardial involvement on admission by LVEF (48%) than by the Tei index (70%). They concluded that most of the patients with severe dengue had asymptomatic myocarditis, as evident by a deranged Tei index, which improved but did not normalize by the time of discharge. Hence, these groups of patients needed a longer follow-up period.

In 2015, Kirawittaya *et al*[34] conducted a study to characterize the incidence and changes in cardiac function by serial ECHO in 181 DF and DHF patients compared with 35 non-dengue patients. They found that DHF patients who developed plasma leakage had lower cardiac index (2695 *vs* 3188 L/min/m^2, P = 0.003) and higher LV myocardial performance index (0.413 *vs* 0.328, P = 0.021) and systemic vascular resistance (2478 *vs* 1820 dynes.s/cm^{-5}, P = 0.005) compared to those without plasma leakage. Moreover, cardiac dysfunction was most pronounced around the time of plasma leakage. They concluded that transient LV systolic and diastolic dysfunction was common in pediatric DHF patients and related to the severity of plasma leakage.

In the same year, Li *et al*[35] conducted a study to investigate the prevalence of myocarditis using ECG, ECHO, and cardiac enzymes tests in 1782 DHF patients. They found that 201/1782 (11.28%) of the patients had myocarditis. Moreover, the prevalence of myocarditis in non-severe dengue with warning signs and severe dengue was 46.66% compared with non-severe dengue without warning signs, which was 9.72%. DHF patients with myocarditis experienced longer hospitalization than those without myocarditis (7.17 ± 4.64 *vs* 5.98 ± 2.69, P = 0.008). They concluded that the incidence of myocarditis increased with the severity of DHF; moreover, the prognosis of DHF patients with and without myocarditis had no significant difference, even if myocarditis patients experienced longer hospital stay.

In 2020, Bhatt *et al*[16] conducted a prospective observational study to evaluate the cardiac involvement among 182 admitted DHF patients of age ≥ 14 years. They found that 44/182 (24%) of the DHF patients demonstrated some ECG abnormalities, such as sinus tachycardia, sinus bradycardia, and non-specific ST changes. Moreover, cardiac markers were elevated in 27/182 (14.8%) of the DHF patients, and among these patients, 11/27 (40.7%) had ECHO evidence of myocarditis. The incidence of myocarditis in that study was 7.10%. The authors concluded that myocarditis among

admitted dengue patients is not uncommon and may cause increased morbidity and mortality. Thus, there is a need to have a CPG to diagnose and treat myocarditis among DHF patients.

Bhattacharjee *et al*[36] conducted a study to compare cardiac markers in DF patients with and without myocarditis. The patients were classified as cases (ECG and ECHO changes of LVEF < 50%; n = 41) and controls (age and sex-matched with normal ECG and ECHO; n = 43). They found that creatine kinase (CK), CK-MB, troponin T, and NT-proBNP concentrations were higher in the cases than in the controls. Serum troponin T and NT-proBNP were also found to be positively correlated to each other. They concluded that routine monitoring of cardiac markers, in conjunction with ECG and ECHO in early diagnosis and management of myocarditis, was needed for DVI patients.

In 2021, Cabrera-Rego *et al*[37] conducted a study to characterize the cardiovascular manifestations in 427 hospitalized patients with DVI. They found that 19.7% of these patients had cardiovascular manifestations. The manifestations were, specifically, sinus bradycardia (13.8%), atrial extrasystoles (4.9%), and ventricular extrasystoles (4.0%). Pericarditis and myocarditis were detected in 1.6% and 0.2%, respectively. The higher probability of presenting cardiac dysfunctions in DVI patients was decreased platelet count [odds ratio (OR = 1.13)], advanced age (OR = 1.70), male sex (OR = 1.94), and dengue with warning signs (OR = 3.29). They concluded that cardiovascular signs in patients with dengue are common and self-limited. It is essential to consider these findings and not underestimate cases with asymptomatic pericarditis or myocarditis.

Shah *et al*[38] conducted a study to evaluate the incidence and spectrum of cardiac abnormalities in 320 patients with DF. They found that 112/320 (35%) of DF patients had changes of cardiac manifestations, with 19.7% having sinus bradycardia, 13.1% having LVEF < 40%, 15% having elevated troponin T, 10.6% having elevated CK-MB, and 5.9% having elevated NTproBNP. The mortality rate was 4.38% among the entire patient population that presented cardiac involvement. They concluded that cardiac dysfunction in patients with DVI was common. Moreover, their findings showed that the abnormalities of ECG, ECHO, and cardiac markers were predictors of risk for adverse outcomes.

HISTOPATHOLOGICAL FINDINGS

An extensive series of histopathological findings regarding DHF patients were reported since 1967[15]. In 1968, Burke[39] reported the autopsy findings of 12 DHF cases, all of which had cardiac involvement, including epicardial petechial hemorrhage, myocardium focal hemorrhage, and subendocardial hemorrhage in the posterior wall of the LV. Moreover, the author stated having not found evidence of myocarditis with normal blood vessels. In 2011, Weerakoon *et al*[20] reported that 5 autopsy DSS patients' histopathological findings showed evidence of florid myocarditis; the distinct histological findings of the myocardium were interstitial edema with inflammatory cell infiltration, necrosis of myocardial fibers, and evidence of pericarditis. They concluded that primary cardiac failure due to myocarditis in DHF is usually overlooked.

Management guidelines for DHF should address myocarditis as an important issue. The primary histopathological features are shown in Table 3[15,20,39-42]. Moreover, DENV antigen has been detected with immunohistochemistry in fatal dengue with clinical myocarditis[13,20,31,43,44]. Myocardial damage had also been demonstrated in patients dying of dengue[20,45]. Weerakoon *et al*[20] showed severe myocardial damage with hemorrhage, interstitial edema with inflammatory cell infiltration, and necrosis of myocardial fibers. Moreover, Torres *et al*[46] demonstrated focal and diffuse myocarditis with cellular infiltration by lymphocytes, neutrophils, and eosinophils in the patients who died from dengue. Pericarditis, characterized by chest pain, has also been demonstrated in histopathology of DHF patients' autopsies[20,47]. This evidence may suggest that direct viral-induced cardiac tissue damage does take place.

PATHOPHYSIOLOGY OF CARDIAC DYSFUNCTION IN DENGUE

Although the exact mechanisms leading to cardiac dysfunction in DVI are not clearly understood, two mechanisms of cardiac injury may cause it, either directly (*via* DENV invasion and damage to the cardiac tissues) or indirectly (*via* cytokine-induced

Table 3 Primary histopathological features of cardiac involvement in autopsy of dengue hemorrhagic fever patients

Ref.	Autopsy cases, *n*	Gross findings	Histopathological findings
Bhamarapravati *et al* [15], 1967	100	Focal areas of hemorrhage in the epicardium, sub endocardium, interstitial tissues, or combined	Separation of myocardial fibers with the interstitial tissue edema. Extensive hemorrhage in myocardium with condensation and acidophilic pattern of myocardial fibrils
Burke[39], 1968	12	Petechial hemorrhage in epicardium, sub endocardium, and myocardium	No evidence of myocarditis. Normal blood vessels
Weerakoon *et al*[20], 2011	5	Evidence of florid myocarditis	Interstitial edema with inflammatory cell infiltration and necrosis of myocardial fibers. Evidence of pericarditis
Kularatne *et al*[40], 2018	3	Petechial hemorrhage	Interstitial edema with infiltration of neutrophils and lymphocytes. Lymphocytic infiltration in endomyseum
Marques *et al*[41], 2013	1	NR	Acute myocarditis with multiple areas of myocardial necrosis surrounded by inflammatory cellular infiltrate
Rathi *et al*[42], 2013	6	Hemorrhagic spots over pericardium, epicardium. Mild ventricular hypertrophy	Lymphocytic myocarditis

NR: Not reported.

immune damage), or both may be at play[20,48]. Numerous inflammatory mediators, such as cytokines, tumor necrosis factor-α, interleukins [(ILs), *e.g.*, IL-1, IL-2, IL-6, IL-13, and IL-18] and cytotoxic factors, as well as increased capillary permeability, may play a vital role in the pathogenesis of cardiac dysfunction[49-51].

Cardiac dysfunction in DVI, although mild and self-limited, can be caused by SDD, which results in progressive and intractable acute heart failure with global hypokinesia and acute cardiac dilatation[24,28,29]. After DENV enters the body, it will be attacked by macrophages, which in turn causes T cell activation. Activated T cells then release several inflammatory mediators and induce the complement system pathways' activation (*i.e.* C3a and C5a). This process will cause inflammation and necrosis of the endothelial cell lining of blood vessels, leading to increased capillary permeability and vascular leakage. When the vascular leakage occurs in myocyte interstitial spaces, it will cause myocardial interstitial edema, leading to the impairment of myocardial contraction and reduced overall function. Moreover, intravascular blood volume decreases, leading to coronary circulation impairment. The release of numerous cytokine mediators also affects cardiac electrical conduction and suppresses cardiac contractility, leading to conduction block and arrhythmias. Another factor that may contribute to cardiac dysfunction in DHF patients is a derangement of calcium ion storage in the myocytes, directly leading to myocarditis [13,52,53].

MANAGEMENT

Cardiac dysfunction in DHF is primarily asymptomatic and transient, and there are no specific guidelines available for its management. Thus, routine ECG will provide clear evidence of myocarditis or abnormal cardiac rhythm in this group of patients. The patients who have abnormal ECG with clinical manifestations of cardiac involvement should be progressed to ECHO evaluation. The most critical problem has been determining whether myocarditis is playing a contributory role in a patient with shock. The goal of the management is to provide symptomatic and supportive care to maintain hemodynamic stability. These goals include maintenance of optimal intravascular volume, with use of diuretics and inotropic drugs as necessary. Moreover, special care should be taken not to cause iatrogenic fluid overload[54,55].

Corticosteroid treatment in SDD with myocarditis remains controversial. Only one study to date has shown rapid recovery of a child with myocarditis upon intravenous administration of methylprednisolone[56]. Tachyarrhythmias, including atrial fibrillation (AF), have also been documented in patients with dengue but are much less common than bradyarrhythmias. A few studies have supported the use of anti-arrhythmic drugs in non-self-limiting AF[57-59]; although, the CPG has still not been clearly defined. However, the efficacy of channel blockers and β-blockers for AF treatment caused by DVI is not conclusive because of co-existing hypotension.

Notably, close and regular monitoring of the clinical presentation of DHF patients is crucial. Furthermore, a vigorous treatment regimen to maintain normal hemodynamic stability is the critical point of DHF patient management.

CONCLUSION

Cardiac dysfunction has been reported to be more severe in patients with DSS than in those with DF or non-shock DHF. The pathophysiology of myocardial damage in DHF patients is not yet fully elucidated. It may result either from direct DENV damage of the cardiomyocytes or an indirect cytokine-mediated immunological response, or even a combined effect. Cardiac involvement in DHF is not uncommon. Although acute, transient and self-limited, acute cardiac dysfunction in DHF may lead to clinically severe state and a fatal outcome. Early detection of cardiac involvement in DVI patients, prompt resuscitation of hemodynamic instability, avoidance of circulatory overload, and sparing the essential tools for critical management are vital in managing DHF patients with severe cardiac dysfunction.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 June 28; 9(3): 297-308

DOI: 10.13105/wjma.v9.i3.297

ISSN 2308-3840 (online)

SYSTEMATIC REVIEWS

Glycated haemoglobin reduction and fixed ratio combinations of analogue basal insulin and glucagon-like peptide-1 receptor agonists: A systematic review

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Conflict-of-interest statement: No conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution

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Abstract

BACKGROUND

Fixed ratio combinations (FRCs) of analogue basal insulin and glucagon-like peptide-1 receptor agonists are a newer addition to the therapeutic armamentarium for the management of type 2 diabetes mellitus. They reduce treatment complexity by combining two injectables in a single daily injectable, thus potentially improving adherence and persistence. Clinicians wanting to use FRCs would need to choose between members of the class.

AIM

To describe and contrast the glycated haemoglobin reduction of two FRCs of analogue basal insulin and glucagon like peptide-1 receptor agonist in adults with type 2 diabetes mellitus.

METHODS

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Manuscript source: Invited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: South Africa

Peer-review report's scientific quality classification

Grade A (Excellent): A, A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E, E

Received: March 27, 2021
Peer-review started: March 27, 2021
First decision: May 12, 2021
Revised: June 5, 2021
Accepted: July 2, 2021
Article in press: July 2, 2021
Published online: June 28, 2021

P-Reviewer: Al-Hadhrami R, Athyros VG, Duan W, Li SY
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Li JH

The following Population, Intervention, Comparison, Outcome question was used for the primary analysis: Among adult patients with type 2 diabetes mellitus [P], what is the effect of iGlarLixi [I] compared to IDegLira [C] for bringing about glycaemic control (as measured by reduction in glycosylated haemoglobin) [O]? The Prisma Statement was used as a guideline for framing this systematic review. We searched PubMed, EMBASE and Cochrane library databases and Clinicaltrials.gov using various keywords and medical search headings related to type 2 diabetes mellitus, iGlarlixi, IDegLira and glycated haemoglobin A1c.

RESULTS

All 14 studies identified by the systematic search met the primary efficacy endpoint of reduction in glycated haemoglobin. There were no head-to-head studies between the FRCs of iGlarlixi and IDegLira, and we therefore did an indirect comparison based on a common comparator of insulin glargine U100. Both iGlarLixi and IDegLira effectively reduce glycated haemoglobin when compared to insulin glargine U100. However, using indirect comparisons, IDegLira had a greater haemoglobin A1c reducing ability (0.6% *vs* 0.3%). The indirect comparison is limited by the differences between the studies; the fasting blood glucose targets were slightly higher for iGlarLixi studies when compared to the IDegLira studies (4.0-5.0 mmol/L and 4.4-5.6 mmol/L), and the IDegLira study used a greater average dose of insulin glargine when compared to the iGlarLixi studies (66 U/d *vs* 40 U/d).

CONCLUSION

Both iGlarLixi and IDegLira effectively reduce glycated haemoglobin. Indirect comparisons, using insulin glargine as the common comparator, suggest that IDegLira reduces glycated haemoglobin to a greater extent than iGlarLixi. However, given the limitations of indirect comparisons, robust head to head studies and real-world data would better inform clinician choice and clinical practice guidelines.

Key Words: Diabetes mellitus; Fixed ratio combinations; Glycated haemoglobin, Glucagon like peptide-1 agonist; Analogue insulin

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Core Tip: This systematic review investigates the effect of fixed ratio combinations of analogue insulin and glucagon like peptide-1 agonists on reduction of glycated haemoglobin. This systematic review helps fulfil a data gap that will help clinicians decide on comparative efficacy of members of the fixed ratio combination class.

Citation: Naidoo P, Bouharati C, Rambiritch V, Karamchand S, Tafuto BA, Leisegang RF. Glycated haemoglobin reduction and fixed ratio combinations of analogue basal insulin and glucagon-like peptide-1 receptor agonists: A systematic review. *World J Meta-Anal* 2021; 9(3): 297-308
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/297.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.297

INTRODUCTION

Diabetes mellitus is a heterogeneous disorder of carbohydrate, protein and fat metabolism, characterized by hyperglycaemia secondary to defects in insulin secretion, insulin action or both[1]. In 2019, approximately half a billion patients were living with diabetes, and this number is projected to increase to 700 million by 2045[2]. Globally, diabetes is the 9th most common cause of death[3], and 9.3% of adults aged 20-79 years have diabetes[4]. The economic impact of managing diabetes and its complications are significant, with an estimated global gross domestic product cost of 2.2% by the year 2030[5].

The majority of patients with diabetes can be classified as having either type 1 (± 5%-10%) or type 2 (± 90%-95%) diabetes mellitus[1]. Good glycaemic control prevents microvascular and macrovascular complications in patients with diabetes[6]. Despite the vast armamentarium of therapies that include oral antidiabetic agents and injectables, attainment of glycaemic control remains suboptimal, and the World Health Organization lists diabetes mellitus as a top 10 cause of death[7,8]. Managing adults with diabetes cost US$1.31 trillion globally in 2015[9]. Beyond the medical complications of diabetes, patients may also be negatively impacted from an emotional, psychological and quality of life perspective[10].

The reasons for non-attainment of glycaemic goals are multifactorial and include complexity of treatment regimens and multiple injections. To reduce complexity and the number of daily injections, fixed ratio combinations (FRCs) of analogue basal insulin and glucagon-like peptide-1 (GLP-1) receptor agonists have recently been added[11]. There are currently two FRCs that are marketed: IGlarLixi and IDegLira. Both have the same mode of action, *i.e.* the analogue basal insulin component increases cellular uptake of glucose and reduces hepatic glucose production, while the GLP-1 receptor agonist stimulates insulin release and inhibits glucagon release[11]. FRCs reduce haemoglobin A1c (HbA1c) by approximately 0.5%[12], and their most common adverse effects are gastro-intestinal events (nausea, vomiting), nasopharyngitis and hypoglycaemia[13,14]. Both are indicated for the management of patients with type 2 diabetes mellitus not controlled on lifestyle modification.

Given the high cost of FRCs and their recent market introduction, there are not many clinical practice guidelines that have assessed them for inclusion. For instance, the World Health Organization[15] and International Diabetes Federation[16] guidelines on diabetes mellitus do not currently include FRCs. In the diabetes field, the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) are leading the path with the publication of a joint guideline for the management of diabetes mellitus which includes FRCs[17]. FRCs have been positioned for patients who are on both GLP-1 receptor agonists and basal analogue insulin.

The joint ADA/EASD consensus statement on the management of patients with type 2 diabetes mellitus does not differentiate between the two FRCs[17]. Clinicians who wish to use FRCs need to consider the body of evidence before choosing between the two marketed products. However, the guideline does not differentiate between the two currently marketed FRCs, and there is no systematic review to assist clinicians decision making. The only systematic review and meta-analysis compare efficacy of FRCs with other classes of anti-diabetic treatments, but none compare iGlarLixi with IDeglira[18]. Both are administered *via* a once daily subcutaneous injection and present similar adverse effects of hypoglycaemia, nasopharyngitis, nausea and vomiting[13,14]. The average United States cost of a month supply of iGlarLixi and IDegLira is $851.09 and $1245.96, respectively[19,20]. It is important to investigate the efficacy of iGlarLixi compared to IDegLira since it may guide the clinicians when making their decision.

The aim of this systematic review is to describe and contrast the glycated haemoglobin reduction of two FRCs of analogue basal insulin and GLP-1 receptor agonist in adults with type 2 diabetes mellitus.

MATERIALS AND METHODS

The review protocol for this systematic review has not been registered. The following Population, Intervention Comparison, Outcome (PICO) question was used for the primary analysis: Among adult patients with type 2 diabetes mellitus [P], what is the effect of iGlarLixi [I] compared to IDegLira [C] for bringing about glycaemic control (as measured by reduction in glycosylated haemoglobin) [O]?

The preferred reporting items for PRISMA Statement was used as a guideline for framing this systematic review[21].

Eligibility criteria

Clinical trials and observational studies investigating the efficacy of FRCs in patients with type 2 diabetes mellitus were identified. We included observational studies to get a sense of the real-world efficacy of FRCs.

Study inclusion criteria were: (1) Male or female, age ≥ 18 years; (2) Subjects diagnosed with type 2 diabetes mellitus; (3) Outpatients receiving treatment with FRCs of iGlarLixi or IDegLira; (4) HbA1c 7.0%-11.0% (both inclusive) (53-97 mmol/mol) by central laboratory analysis; (5) Body mass index ≥ 20 kg/m^2 and < 40

kg/m^2; (6) Randomised clinical trial or observational study; (7) At least 10 patients per each study group; (8) Dropout rate < 20%; (9) Typically, if an author is included on more than one primary research article that is similar in content, the most recent review or article will be accepted and earlier versions will be rejected; (10) If an author is included on more than one primary research article and the content is different, then both reviews may be accepted; and (11) Studies published in English language.

Study exclusion criteria consisted of: (1) Patients < 18 years; (2) HbA1c > 11%; (3) Hospitalized; (4) History of pancreatic cancer; (5) Renal failure (estimated glomerular filtration rate < 30 mL/min); (6) Liver failure or impairment defined as alanine aminotransferase or aspartate aminotransferase ≥ 2.5 times upper limit of normal; (7) Screening calcitonin ≥ 50 ng/L; (8) Type 1 diabetes mellitus; (9) History of pancreatitis (acute or chronic); (10) Personal or family history of medullary thyroid carcinoma or multiple endocrine neoplasia type 2; (11) Subjects presently classified as being in New York Heart Association Class IV; (12) Screening calcitonin ≥ 50 ng/L; (13) Currently pregnant or breastfeeding or not using a reliable method of birth control for the duration of the trial in all females with childbearing potential; (14) < 10 subjects per intervention group; (15) Studies of less than 3-mo duration; (16) Dropout rate > 20%; and (17) Studies not reported in English.

Search strategy and study selection

Sixty-six articles were identified by searching PubMed, Embase and Cochrane library databases as well as Clinicaltrials.gov using various keywords and medical search headings (MeSH) related to type 2 diabetes mellitus, iGlarlixi, IDegLira and glycated HbA1c. The complete search syntax conducted on March 21, 2021 was as follows: (1) Patient ((((((((((((((type 2 diabetes mellitus[MeSH Terms]) OR adult-onset diabetes mellitus) OR ketosis-resistant diabetes mellitus) OR maturity-onset diabetes mellitus) OR non-insulin dependent diabetes mellitus) OR non-insulin dependent diabetes mellitus) OR noninsulin dependent diabetes mellitus) OR noninsulin-dependent diabetes mellitus) OR slow-onset diabetes mellitus) OR stable diabetes mellitus) OR type II) OR MODY) OR maturity-onset diabetes)) OR maturity onset diabetes mellitus)) OR NIDDM) OR type 2 diabetes))); (2) Outcome ((((((((((((((((((((glycated hemoglobin[MeSH Terms]) OR (a) OR glycated hemoglobin a1c)) OR OR glycated haemoglobins) OR OR glycohemoglobin a) OR OR glycosylated hemoglobin a) OR OR glycosylated hemoglobin a1c) OR OR hb A1) OR OR Hb A1a+b) OR OR Hb A1a-1) OR OR hb A1a-2) OR OR hb a1b) OR OR hb A1c) OR OR HbA1) OR OR hemoglobin A(1)) OR hemoglobin A)) OR OR glycosylated) OR OR hemoglobin, glycated A1a-2) OR OR hemoglobin, glycated A1b) OR OR hemoglobin, glycosylated) OR OR hemoglobin, glycosylated A1a-1) OR OR hemoglobin, glycosylated A1b)); (3) (1) AND (2); (4) Intervention ((((((glarlixi[MeSH Terms]) OR insulin glargine/lixisenatide) OR (insulin glargine and lixisenatide)) OR soliqua)); (5) Comparator (((((ideglira[MeSH Terms]) OR insulin degludec/Liraglutide) OR (liraglutide and insulin degludec)) OR xultrophy) OR xultrophy 100/3.6)); and (6) (4) OR (5); and (7) (3) AND (6).

Of the 66 articles that were screened (duplicate n = 0), 52 articles were excluded for the following reasons: Post-hoc analysis (n = 11), review article (n = 21), did not contain FRC (n = 9), retrospective chart review (n = 1), mathematical model (n = 1), pharmacokinetic model (n = 1), case study (n = 1), cost-effectiveness study (n = 1), type III diabetes mellitus (n = 1), study duration less than 3 mo (n = 1) and animal study (n = 1). The remaining 14 articles were used for qualitative synthesis. The PRISMA flow diagram outlining the search process used is provided in Figure 1.

Data collection

A PICO tracker was used for data extraction. We included key elements from each study, *i.e.* country location, clinical trial phase, patient population, intervention, comparison, outcome measure, response assessment day, time-points of study measurements and study design. The two study arms were FRC *vs* comparator.

Bias was assessed using the Cochrane Risk of Bias tool[22]. Studies with seven or greater individual dimensions rated as "High" were assigned an overall "Poor" bias rating, studies with between three and six dimensions rated as "High" were given a "Moderate" overall bias rating and studies with less than three "High" dimensions were given a "Good" overall bias rating.

Data analysis

Since there were no head-to-head studies comparing iGlarLixi with IDeglira, we conducted an indirect comparison. We compared the FRCs if they had a common comparator to ensure that we are comparing "like with like." From the 14 studies

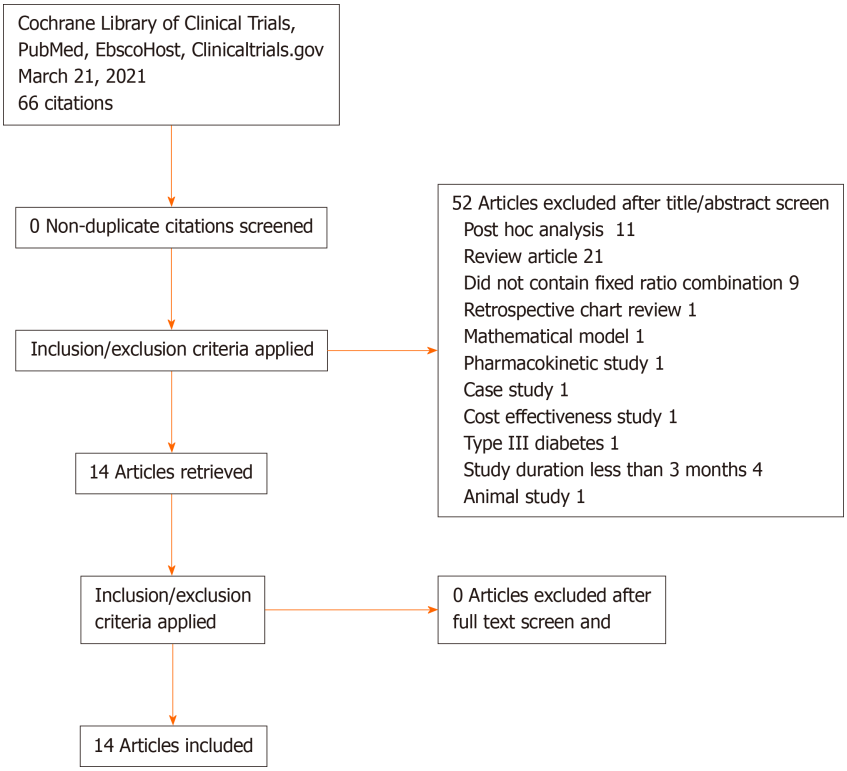


Figure 1 PRISMA flow diagram.

identified, three studies had a common comparison.

Outcome measures

The primary outcome measure was reduction in glycated haemoglobin after at least 6 mo of treatment, as per the aim of this systematic review.

RESULTS

Included studies and study characteristics

In total, 14 studies were identified through the systematic review process. All studies met their primary efficacy endpoint of reduction in glycated haemoglobin. The details of these studies are contained in Supplementary Tables 1 and 2. The majority of the studies were phase III studies, with one phase II study. Except for three studies conducted in Japan, the rest of the studies were multi-country clinical trials. The studies were a minimum of 24 wk and a maximum of 104 wk.

Of the 14 studies identified, none were direct comparisons between FRCs. Therefore, we focused on studies that had a common comparator; these studies totalled three and are listed in Table 1. The common comparator was insulin glargine U100. One study looked at the efficacy of IDegLira on a background of sodium glucose co-transporter inhibitors[23]. We omitted this study because it was not comparable to the iGlarLixi study given the difference in background therapy. We focused on indirect comparisons in which the background therapy was similar; in this case it was the background of metformin and this included studies that were similar and relatively homogenous, thus making indirect comparison of IDeglira and iGlarLixi sensible as per Butchers method[24].

In the phase III multinational DUAL V study, Lingvay *et al*[25] investigated whether IDegLira was non-inferior to up-titration of glargine, with reduction in glycated haemoglobin as the primary efficacy endpoint measured at week 26. Patients had type 2 diabetes and were uncontrolled (HbA1c 7%-10%) despite the use of metformin (≥ 1500 mg/d or maximum tolerated) and insulin glargine (20-50 U/d). Patients were randomised to U100 or IDeglira in a 1:1 ratio. IDeglira was initiated at 16 dose steps (16 U of degludec/0.6 mg of liraglutide). The maximum dose of degludec and liraglutide was 50 U and 1.8 mg, respectively. Patients randomised to glargine continued with their glargine dose, with no maximum allowable dose. Both treatments

Table 1 Study characteristics

Ref.	Study design	Patient population	Intervention	Comparator	Efficacy endpoint	Efficacy outcome time-point
Lingvay *et al*[25]	Multinational, multicentre, 26-wk, randomised, open-label, 2-group, treat-to-target trial	Adults with type 2 diabetes	Insulin degludec/liraglutide	Insulin glargine U100	Baseline HbA1c level was 8.4% for the degludec/liraglutide group and 8.2% for the glargine group. HbA1c level reduction was greater with degludec/liraglutide *vs* glargine [-1.81% for the degludec/liraglutide group *vs* -1.13% for the glargine group; estimated treatment difference, -0.59% (95%CI: -0.74 to -0.45)], meeting criteria for noninferiority ($P < 0.001$) and meeting criteria for statistical superiority ($P < 0.001$)	26-wk
Rosenstock *et al*[26]	Randomised, open-label, parallel group, multicentre	Adults with type 2 diabetes	iGlarLixi	Insulin Glargine U100	Mean HbA1c was reduced from 8.0% at baseline to 6.3% and 6.5% with LixiLan and Gla-100, respectively, establishing statistical noninferiority and superiority of LixiLan [least-squared mean (95%CI) difference: -0.17% (-0.31, -0.04) (-1.9 mmol/mol, -3.4, -0.4); $P = 0.01$]	24-wk
Rosenstock *et al*[28]	Randomised, parallel, open label, 3-arm-treatment	Adults with type 2 diabetes	iGlarLixi	Insulin glargine U100; Lixisenatide	Greater reductions in HbA1c from baseline (8.1%) were achieved with iGlarLixi compared with iGlar and Lixi (-1.6%, -1.3%, -0.9%, respectively), reaching mean final HbA1c levels of 6.5% for iGlarLixi *vs* 6.8% and 7.3% for iGlar and Lixi, respectively (both $P < 0.0001$)	30-wk

CI: Confidence interval; HbA1c: Haemoglobin A1c.

were titrated to achieve a fasting blood glucose of 4.0-5.0 mmol/L. The final dose of insulin glargine and insulin degludec was 66 U and 41 U, respectively. At week 26, HbA1c had decreased by 1.81% for the IDegLira group (standard deviation 1.08%) and by 1.13% for the glargine group (standard deviation 0.98%); the estimated treatment difference was of 0.59% [95% confidence interval (CI): -0.74-0.45; $P < 0.001$] and was clinically and statistically significant. Further details of the DUAL V study are contained in Tables 1-3.

In a phase II, proof-of-concept, randomised, open label study, Rosenstock *et al*[26] investigated the safety and efficacy of iGlarLixi compared to insulin glargine U100 in insulin naïve patients with uncontrolled type 2 diabetes (HbA1c ≥ 7% to ≤ 10%) on a background of metformin (≥ 1500 mg/d for ≥ 3 mo). The primary efficacy endpoint was a reduction in HbA1c at week 24. The starting dose was of 10 U of iGlarLix and 10 U in the U100 group. iGlarlixi and U100 were titrated based on a fasting blood glucose target of 4.4-5.6 mmol/L. The maximum daily dose of iGlarLixi was 60 units U100, which corresponded to a lixisenatide dose of 30 μg. There was no upper limit for the dose of glargine U100. The mean baseline HbA1c ranged from 8.0% to 8.1%. IGlarLixi and insulin glargine U100, resulted in reduction in HbA1c of 1.82% and 1.64%, respectively. The difference between mean change from baseline for iGlarLixi and insulin glargine U100 was -0.17% ($P = 0.01$). The average dose of insulin glargine U100 was 39 U at week 24.

As a follow up of the above proof-of-concept study, Rosenstock *et al*[26] conducted a multinational, randomised, open label phase III study in which iGlarLixi was compared to its components, *i.e.* insulin glargine U100 and lixisenatide. The primary efficacy endpoint was change in HbA1c at week 30. Adults with type 2 diabetes who were uncontrolled on metformin (HbA1c ≥ 7.5% and ≤ 10%) or metformin in combination with other oral antidiabetic agents (HbA1c ≥ 7.0% and ≤ 9.0%) were included. Patients on metformin and second oral agent were asked to discontinue the second oral agent during the run-in phase. During the run-in phase metformin was titrated to at least 2000 mg or the maximum tolerated dose of at least 1500 mg/d. After the run in, patients with an HbA1c of ≥ 7.0% and ≤ 10.0% and fasting plasma glucose ≤ 13.9 mmol/L were randomised to one of the three arms in a 2:2:1 ratio (iGlarLixi; insulin glargine U100; lixisenatide). iGlarLixi and insulin glargine U100 was started at 10 U/d with the maximum allowed dose of 60 U/d. Lixisenatide was started at 10 μg for the first 2 wk and then 20 μg for the rest of the study period. The final mean basal insulin daily dose was 39.8 U and 40.3 U for iGlarLixi and insulin glargine U100, respectively. The baseline HbA1c was 8.1% in all three groups, and mean HbA1c at week 30 were 6.5%, 6.8% and 7.3% for iGlarLixi, insulin glargine U100 and

Table 2 Patient arm characteristics

		Baseline characteristics															
		Gender	Ethnic origin										OAD at screening				
Ref.	Arms	M (%)/F (%)	White (%)	Black (%)	Asian (%)	Other (%)	Age (yr)	Bodyweight	BMI (kg/m^2)	Duration of Diabetes (yr)	HbA1 (%)	Hb (mmol/mol)	FPG (mmol/L)	Metformin	Metformin plus pioglitazone	Sulphonylurea	Other
Lingvay *et al*[25]	Degludec/liraglutide (*n* = 278)	51.4/48.6	94.2	2.2	3.2	0.4	58.4	88.3	31.7	11.64	8.4	NA	8.9	NA	NA	NA	NA
	Glargine (*n* = 279)	49.1/50.9	95.0	1.8	3.2	0.0	59.1	87.3	31.7	11.33	8.2	NA	8.9	NA	NA	NA	NA
Rosenstock *et al*[26]	LixiLan (*n* = 161)	49.7/50.3	98.1	NA	NA	NA	56.9	90.1	32.2	6.3	8.1	64	9.8	Yes	NA	NA	NA
	Gla-100 (*n* = 162)	52.5/47.5	98.8	NA	NA	NA	56.6	91.6	32.0	7.1	8.0	64	9.5	Yes	NA	NA	NA
Rosenstock *et al*[28]	iGlarLixi (*n* = 469)	47.3/52.7	88.9	7.0	1.7	2.3	58.2	NA	31.6	8.9	8.1	65	9.9	Yes	NA	55.2	Glinide, Sodium glucose co-transporter inhibitor, dipeptidyl dipeptidase 4 inhibitor
	iGlar (*n* = 467)	50.7/49.3	90.1	7.1	1.5	1.3	58.3	NA	31.7	8.7	8.1	65	9.8	Yes	NA	53.3	
	Lixi (*n* = 234)	56.8/43.2	92.3	5.1	1.3	1.3	58.7	NA	32.0	8.9	8.1	65	9.8	Yes	NA	52.6	
	All (*n* = 1170)	50.6/49.4	90.1	6.7	1.5	1.7	58.4	NA	31.7	8.8	8.1	65	9.8	Yes	NA	53.9	

NA: Not available; BMI: Body mass index; M: Male; F: Female; Hb: Haemoglobin; FPG: Fasting plasma glucose; OAD: Oral antihyperglycaemic drug.

lixisenatide, respectively. The HbA1c difference at week 30 between iGlarLix and insulin glargine U100 was -0.3% (95%CI: -0.4% to -0.2%, $P < 0.0001$).

Patient characteristics

All participants were adults with type 2 diabetes with a disease duration of 7-11 years. Both genders were included in the studies, and the majority of participants were Caucasian (89%-98%). Patients were obese (31-32 kg/m^2) with a baseline glycated haemoglobin of approximately 8.0%-8.4%. The patient characteristics are listed in Table 2.

Arm characteristics and interventions

Given that treatment was randomly allocated, the intervention and comparator arms were balanced with respect to baseline characteristics and glycated haemoglobin. The interventions were the FRCs compared with insulin glargine U100.

Outcomes

The primary efficacy outcome was reduction of glycated haemoglobin assessed after at least 24 wk. We did not assess secondary efficacy outcomes of reduction in fasting and postprandial glucose levels. We also did not assess safety as this was beyond the scope of this systematic review.

Risk of bias

Risk of bias and study quality were assessed for each included study using Cochrane Risk of Bias tool[22]. Differences in rating were resolved *via* discussion and consensus among all authors. The quality assessment for each study is contained in Table 3. The studies were industry sponsored randomised phase II-III studies with a moderate risk of bias. The major limitation was the open-label design.

DISCUSSION

This systematic review described the ability of FRCs to reduce glycated haemoglobin. Both iGlarLixi and IDeglira reduced HbA1c and met their primary efficacy endpoints in the 14 clinical trials identified in this systematic review. Our findings are similar to those of the systematic review and meta-analysis of Liakopoulou *et al*[27] who showed effective glycaemic control with FRCs when compared with each individual component alone (change in HbA_{1c} -0.31%; 95%CI: -0.47 to -0.16; I^2 = 81 and -0.73%; 95%CI: -0.87 to -0.58; I^2 = 74% compared with basal insulin and GLP-1 RA, respectively).

The studies identified in our systematic review were well designed in general with a moderate risk of bias as assessed by the Cochrane risk of bias tool[22]. The key limitation of the studies was the use of an open label design in which both study participants and investigators were not blinded to interventions.

Given the lack of head-to-head studies of iGlarLixi and IDeglira, we used an indirect comparison to get a sense if there were differences in HbA1c reducing ability of the FRCs. There were three studies in which insulin glargine U100 was the common comparator[25,26,28]. IDegLira reduced glycated haemoglobin to a greater extent than iGlarLixi (approximately 0.6% *vs* 0.3%). However, indirect comparisons have challenges as the studies do have differences that do not allow for firm conclusions. For example, although the studies were treat-to target studies, the target ranges were different with IDegLira and iGlarLixi being 4.0-5.0 mmol/L and 4.4-5.6 mmol/L, respectively[25,28]. Furthermore, the IDegLira study used greater average doses of glargine when compared to the iGlarLixi studies (66 U/d *vs* 40 U/d)[25,28].

Based on indirect comparison, the difference in HbA1c reduction may be a chance finding or due to inherent differences between the studies, *e.g.*, different countries, different investigators and varying doses. Furthermore, there is no biological plausibility that would explain the HbA1c reducing difference between iGlarLixi and IDeglira.

Glycated haemoglobin is only a validated surrogate measure of microvascular complications (retinopathy, nephropathy, neuropathy)[29-37]; ideally one would need to have studies that determine the effect of FRCs on major adverse cardiovascular events, *e.g.*, myocardial infarction, cerebro-vascular accident, peripheral vascular disease and ultimately mortality.

Although the studies were of reasonable duration (at least 24 wk), one would require long term studies to determine the durability of glycated haemoglobin. Ideally one would need a head to head study to determine the differences in safety, efficacy and tolerability between the two FRCs. However, it is unlikely that this would be done given the high costs of doing such a study. Perhaps real-world evidence may help differentiate between the FRCs. A network meta-analysis may assist with informing the relative efficacy and safety of the FRCs. Pharmacoeconomic considerations may also help differentiate between these agents.

Clinical trials help inform clinical practice guidelines. However, there are many factors influencing the translation of clinical practice guidelines to clinical practice. Factors include the level of evidence and the grade of recommendation, the credibility and expertise of the guideline committee, economic factors and physician and patient preference. Given the current lack of head-to-head studies between iGlarLixi and IDeglira, it is not surprising that the joint ADA/EASD guideline[15] does not differentiate between the individual FRCs. It is unlikely that this systematic review would result in a change of the clinical trial guidelines given that it only looks at differences in reduction of glycated haemoglobin and is also an indirect comparison, with its

Table 3 Study bias assessment

Ref.	Selection bias: Random sequence generation	Selection bias: Allocation concealment	Performance bias: Blinding of participants	Performance bias: Blinding of personnel/care providers	Detection bias: Blinding of outcome assessor	Attrition bias: Incomplete outcome data	Reporting bias: Selective outcome reporting	Intention-to-treat-analysis	Selection bias: Group similarity at baseline	Performance bias: Co-interventions	Performance bias: Compliance	Detection bias: Timing of outcome assessments	Additional bias	Overall quality
Lingvay *et al*[25]	L	H	H	H	L	L	L	L	L	L	L	L	L	Moderate
Rosenstock *et al*[26]	L	H	H	H	L	L	L	L	L	L	L	L	L	Moderate
Rosenstock *et al*[28]	L	H	H	H	L	L	L	L	L	L	L	L	L	Moderate

H: High risk of bias; M: Moderate risk of bias; L: Low risk of bias.

inherent limitations.

CONCLUSION

Both iGlarLixi and IDegLira effectively reduce glycated haemoglobin. Indirect comparisons, using insulin glargine as a common comparator, indicate that IDegLira reduces glycated haemoglobin to a greater extent than does iGlarLixi. However, given the limitations of indirect comparisons, robust head to head studies and real-world data are needed to inform clinical practice guidelines.

ARTICLE HIGHLIGHTS

Research background

Fixed ratio combinations of insulin and glucagon-like peptide-1 analogues are novel therapy for the management of patients with type 2 diabetes mellitus.

Research motivation

There is minimal data comparing the fixed ratio combinations of iGlarlixi and IDegLira.

Research objectives
We aimed to compare the glucose lowering effect of iGlarLixi *vs* IDegLira.

Research methods
We used a Population, Intervention, Comparison, Outcome (PICO) question for the primary analysis.

Research results
Both iGlarLixi and IDegLira effectively reduce glycated haemoglobin when compared to insulin glargine U100. However, using indirect comparisons, IDegLira had a greater HbA1c reducing ability (0.6% *vs* 0.3%).

Research conclusions
Both iGlarLixi and IDegLira effectively reduce glycated haemoglobin.

Research perspectives
Head to head studies between iGlarlixi and IDegLira are required to determine if there are clinically relevant differences between the two aforementioned fixed ratio combinations.

ACKNOWLEDGEMENTS

Dr. Roopnarain C for assisting with construction of tables.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 June 28; 9(3): 309-316

DOI: 10.13105/wjma.v9.i3.309

ISSN 2308-3840 (online)

META-ANALYSIS

Impact of *Streptococcus pyogenes* infection in susceptibility to psoriasis: A systematic review and meta-analysis

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Author contributions: Keikha M conceived the study; Keikha M provided critical analyses; Keikha M and Karbalaei M analyzed the data; Yousefi A and Karbalaei M wrote the draft; Keikha M revised the draft; All authors read and approved the final manuscript.

Conflict-of-interest statement: The authors have no conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Psoriasis is one of the most common chronic systemic diseases, mainly appearing as lesions on the skin and joints, and is associated with a high mortality due to a lack of standard treatment. The exact mechanism of this disease is not fully understood, but the inflammatory response and dysregulation of the immune system are the most important molecular processes that trigger this disease. The skin microflora is one of the main factors involved in inducing, maturing, and dysregulating the immune system, which may underly the development of psoriasis.

AIM

To determine the impact of *Streptococcus pyogenes* (*S. pyogenes*) infection and susceptibility to psoriasis using available case-control studies.

METHODS

In this study, we conducted a comprehensive literature search using PubMed, Scopus, Web of science, and Google scholar databases to obtain all available relevant studies on the association between *S. pyogenes* and psoriasis. We pooled the data using Comprehensive Meta-analysis software to investigate the role of *S. pyogenes* infection in the development of psoriasis. The probable connection between *S. pyogenes* and susceptibility to psoriasis was assessed using the odds ratio (OR) with corresponding 95% confidence intervals (CIs).

RESULTS

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Manuscript source: Unsolicited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: Iran

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 23, 2021
Peer-review started: April 23, 2021
First decision: May 19, 2021
Revised: May 24, 2021
Accepted: June 17, 2021
Article in press: June 17, 2021
Published online: June 28, 2021

P-Reviewer: Muthu S, Sun Q
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Li X

Data from 781 cases were evaluated in this study. Our results showed that the rate of infection with *S. pyogenes* in psoriatic patients and healthy individuals was 33.4% (95%CI: 27.8-39.6) and 16.2% (95%CI: 9.7-25.9), respectively. *S. pyogenes* infection significantly increased the risk of psoriasis (OR: 6.58; 95%CI: 3.64-11.87; $P = 0.001$).

CONCLUSION
S. pyogenes infection can significantly increase the risk of psoriasis. Thus, infection with *S. pyogenes* is a risk factor for the initiation and development of psoriatic events.

Key Words: Meta-analysis; Psoriasis; *Streptococcus pyogenes*; Infection; Susceptibility

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Core Tip: Currently, the causes of autoimmune diseases are being seriously considered. According to various studies, both genetic and epigenetic events are reasonable hypotheses for these diseases. Psoriasis is one of the most common autoimmune diseases, and studies conducted in recent decades have shown that infectious diseases caused by *Streptococcus pyogenes* (*S. pyogenes*) (*e.g.*, pharyngitis) may be associated with psoriasis. Based on these studies, autoantibodies produced against streptococcal M12 protein potentially react with human keratin. Statistical analyses in this study showed that infection with *S. pyogenes* increased the risk of psoriasis. Thus, long-term treatment as well as strategies to prevent streptococcal infections will be effective in reducing the risk of psoriasis.

Citation: Yousefi A, Karbalaei M, Keikha M. Impact of *Streptococcus pyogenes* infection in susceptibility to psoriasis: A systematic review and meta-analysis. *World J Meta-Anal* 2021; 9(3): 309-316
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/309.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.309

INTRODUCTION

Psoriasis is one of the most prevalent multisystem chronic cutaneous inflammatory diseases, usually characterized by lesions including red, dry, itchy, and scaly plaques on the elbows, knees, scalp, and lower back[1]. This disease occurs at all ages and in all parts of the world[1,2]. Based on recent studies, the prevalence of psoriasis is estimated to be 0%-2.1% in children and 0.91%-8.5% in adults[2]. According to the World Health Organization, the prevalence of psoriasis in different countries is reportedly 0.09%-11.43%, and 100 million people worldwide suffer from this disease annually[3]. This disease affects the quality of life of patients, as well as their physical activities, and emotional and social health. Unfortunately, there are no specific and effective treatments for this disease[2-4].

The underlying cause of psoriasis is not yet fully recognized. However, several factors such as smoking, stress, alcohol consumption, genetics, hormones, and infectious agents can affect the susceptibility of people to psoriasis[5]. Meanwhile, the role of infectious agents in the development of psoriasis is prominent, as the microbiome can dysregulate the immune response by stimulating the inflammatory process, which triggers the development of autoimmune diseases, particularly psoriasis[1-3]. According to the literature, infectious agents such as *Staphylococcus aureus*, *Streptococcus pyogenes* (*S. pyogenes*), *Propionibacterium acnes*, *Helicobacter pylori*, and *Candida spp.* can induce psoriasis[5,6]. In general, *Streptococci* are a predominant microbiome of the human skin and mucous membrane in the healthy population that can cause numerous opportunistic infections[7]. This group of bacteria contains a variety of enzymes, toxins, and superantigens that can stimulate the immune system and cause psoriasis[8].

The possible interaction between previous infection with *S. pyogenesis* and the development of psoriasis remain unknown. In addition, the potential association

between *S. pyogenes* and psoriasis has been contradictory in some previously published studies. Hence, for the first time, we conducted this meta-analysis to discuss the molecular aspects of *S. pyogenes* infection in the development of various psoriatic events using available case-control documents.

MATERIALS AND METHODS

Search strategy

We conducted a systematic search using global databases such as PubMed (available at: https://pubmed.ncbi.nlm.nih.gov/), Scopus (Available at: https://www.scopus.com/search/form.uri?display=basic#basic), Web of Science online databases (available at: https://mjl.clarivate.com/search-results), and Google scholar (available at: https://scholar.google.com/) to collect all of the studies related to the possible association between *S. pyogenes* infection and psoriasis published up to October 2020 without time limits. In this regard, two authors independently searched related articles using the keywords "*Streptococcus pyogenes*," "*Streptococcus* group A," "*S. pyogenes*" and "psoriasis" according to medical subject heading terms.

Selection criteria

The initial documents collected met the criteria for selecting eligible documents. Inclusion criteria were: cross-sectional, case-control, and longitudinal studies on the association between *S. pyogenes* and psoriasis; studies with available full-text; studies on the evaluation of infection frequency in the case and control groups; studies on the assessment of *S. pyogenes* infection using reliable methods; and studies published in the English language. However, studies including review articles, letters and case-reports; Congress articles; duplicate studies; full-text articles with unclear methods and results; and full-text with repetitive samples were excluded. Disagreements between two authors were resolved by a third author.

Data extraction and quality assessment

The Newcastle-Ottawa Scale checklist was used to evaluate the quality of eligible articles. Subsequently, the required information such as: the first author, publication year, location of studies, number of patients as well as healthy controls, number of *S. pyogenes* strains in the case/control groups, diagnostic method, and relevant reference number were carefully reviewed by two independent authors. The characteristics of eligible studies are listed in the Table 1.

Statistical analyses

The meta-analysis was conducted using Comprehensive Meta-Analysis software version 2.2 (Biostat, Englewood, NJ, United States). First, we assessed the frequency of *S. pyogenes* infection in both patients with psoriasis (case) and healthy individuals groups with 95%CIs. We used the OR with 95%CIs to evaluate the effect of *S. pyogenes* infection on developing psoriasis. Heterogeneity between studies was determined using the statistical inconsistency and Cochrane *Q*-test ($P < 0.05$). The random-effects model, based on the Dersimonian and Laird method, was used when $I^2 > 25\%$ and Cochrane $P > 0.05$; otherwise, we used the fixed-effects model. In addition, the presence of publication bias was measured using Begg's and Egger's tests[9].

RESULTS

In the initial search, 93 articles were selected. In the next step, duplicate studies were deleted, and finally nine articles were recognized as eligible studies and entered used for quantitative analyses (Figure 1).

In this study, the data were evaluated from 781 cases, including 458 psoriatic patients and 323 healthy individuals. Of the studies included, the number of studies was as follows: two in the United Kingdom, two in Iceland, two in Mexico, one in Pakistan, one in Germany, and one in China[10-18]. These studies were conducted during 1991-2008. In these studies, psoriasis was diagnosed using clinical manifestations or pathology examinations, and infection with *S. pyogenes* was diagnosed using tests such as skin CD4+ T cell-producing interferon gamma (IFN-γ), conventional microbiology, antistreptolysin-O (ASO) tetier, anti-beta-hemolytic *Streptococci*, immuno-fluorescence, and identification of heat shock protein 60 (Table 1).

Table 1 Characteristics of included studies

Ref.	Location	Total cases		Number of streptococcal strains		Diagnostic method	NOS score
		Case	Control	Case	Control		
Baker *et al*[10], 1991	Iceland	78	27	42	0	Antistreptolysin-O	8
Baker *et al*[11], 1993	Iceland	9	18	5	4	Skin TCL	8
Villeda-Gabriel *et al*[12], 1998	Mexico	68	56	14	0	Immunofluorescence	6
Brown *et al*[13], 2000	United Kingdom	13	12	10	5	Skin TCL	7
Cancino-Díaz *et al*[14], 2004	Mexico	218		OR: 11.11; 4.33-28.49		Hsp60	5
Weisenseel and Prinz[15], 2005	Germany	19	9	8	0	Antistreptolysin-0	6
Zhao *et al*[16], 2005	China	98	42	5	1	Conventional	7
Raza *et al*[17], 2007	Pakistan	40	40	5	0	Conventional	8
Nahary *et al*[18], 2008	United Kingdom	24	10	7	3	Anti-beta-hemolytic streptococci	7

Hsp60: Heat shock protein 60; NOS: Newcastle-Ottawa scale; OR: Odds ratio; TCL: T-cell lymphoma.

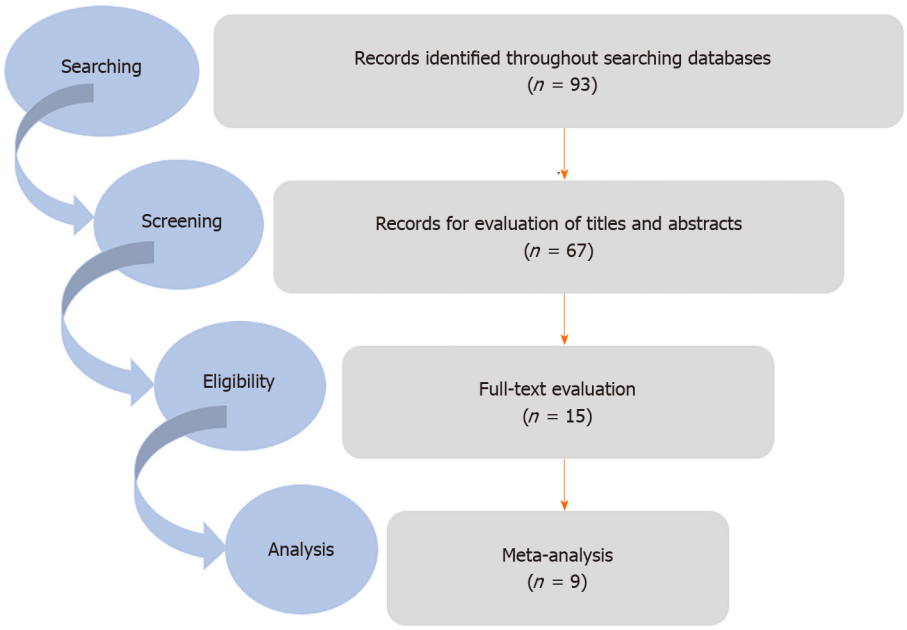


Figure 1 Flowchart of the search strategy and study selection.

According to the results of this study, the rate of infection with *S. pyogenes* in psoriatic patients and healthy individuals was 33.4% (95%CI: 27.8-39.6; $P = 0.001$; $I^2 = 88.36$; $Q = 60.18$; $P = 0.01$; Egger's $P = 0.33$ and Begg's $P = 0.45$) and 16.2% (95%CI: 9.7-25.9; $P = 0.001$; $I^2 = 69.52$; $Q = 22.97$; $P = 0.01$; Egger's $P = 0.04$; Begg's = 0.02), respectively. Moreover, we found that infection with *S. pyogenes* significantly increased the risk of developing psoriasis (OR: 6.58; 95%CI: 3.64-11.87; $P = 0.001$; $I^2 = 33.32$; $Q = 11.99$; $P = 0.15$; Egger's $P = 0.38$; Begg's $P = 0.23$) (Figure 2).

DISCUSSION

Although the exact etiology of psoriasis is not clear, similar to acute rheumatic fever, the onset of clinical manifestations of only type I (but not type II) of psoriasis are

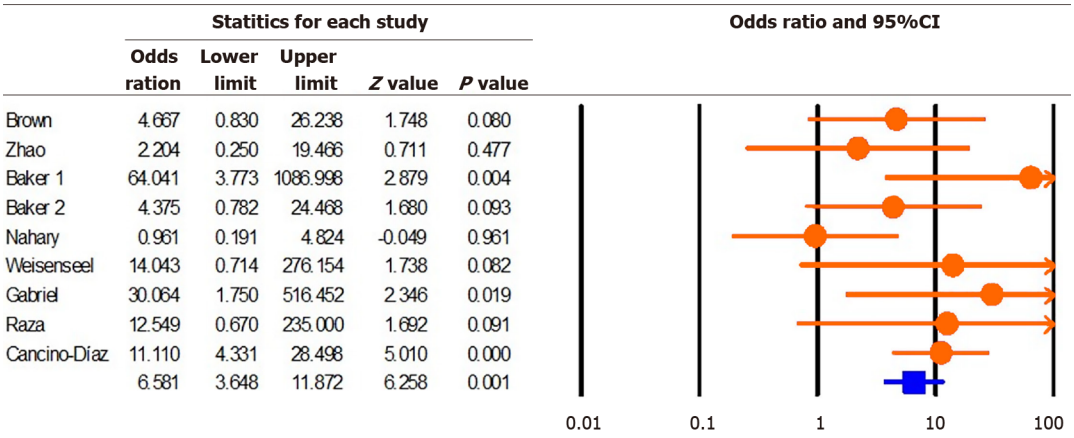


Figure 2 The probable association between *Streptococcus pyogenes* infection and susceptibility to psoriasis. CI: Confidence interval.

associated with pharyngitis caused by *S. pyogenes*[19,20]. However, factors such as hyperproliferation of keratinocytes and dysregulation of the immune system are among the most important aspects that play a key role in the immunopathogenesis of psoriasis. The interaction among the microbiome, keratinocytes, T cell lymphocytes, neutrophils, monocytes, and dendritic cells is highly significant in the development of psoriasis[21,22]. Skin microflora, particularly *Streptococci*, contain several enzymes, toxins, and superantigens that can induce proinflammatory responses, including the production of tumor necrosis factor alpha (TNF-α), IFN-γ, and interleukin 8 (IL-8), as well as production of chemokine receptors, *e.g.*, E-selectin, P-selectin, and CD4 + T cells[23,24]. In addition, recent studies have demonstrated that HLA-Cw*06 effectively presents *Streptococci* antigens to CD8+ cytotoxic T lymphocytes, leading to a balance distribution between T helper type 1 (Th1) and Th2[23-25]. During chronic inflammation, T-cell lymphocytes are recruited to cutaneous lymph nodes in response to IL-8 and chemokine receptors, and hyperproliferation of keratinocytes also occurs due to high levels of TNF-α and IFN-γ[25,26]. In addition, molecular mimicry between *Streptococci* antigens and auto-antigens of normal skin can also induce the production of antibodies that cross-react with some normal skin auto-antigens. In a study by Muto *et al*[27], it was shown that the serum titers of antibodies against the protein M12 (similar to sub-units of human keratin) of *S. pyogenes* were higher in patients with psoriasis than in control subjects[27-29]. The presence of *Streptococci* in the chronic psoriasis plaques indicates the role of these bacteria in increasing the risk of psoriasis [30].

For the first time, Shelley *et al*[31] found that intradermal injection of heat-inactivated group A streptococcus (GAS) in the finger of a 39-year-old woman with psoriasis exacerbated psoriasis skin lesions in her finger. Gross *et al*[32] showed that psoriatic patients have a specific cellular immune response against GAS antigen. In their studies, Baker *et al*[10] found that skin T cells in the psoriasis patients are activated and react with M proteins of *S. pyogenes*. In this study, we found that the infection with *S. pyogenes* significantly increased the risk of psoriasis (OR: 6.58; 95%CI: 3.64-11.87). According to the literature, the rate of infection with *S. pyogenes* is high in psoriatic patients. For instance, El-Rachkidy *et al*[33] demonstrated that a large population of psoriatic patients had high immunoglobulin G titers (> 500) against *S. pyogenes*. According to studies by Tervaert and Esseveld[34], McFadden *et al*[35], and Lilja *et al*[36], the rate of *S. pyogenes* infection in the psoriatic patients is reportedly 88%-97%. Kim *et al*[37] showed that the rate of psoriasis in children is significantly associated with ASO serum levels. It seems that *streptococci* can survive as a facultative intracellular pathogen within the epithelial cells of the tonsils, and as a stable source, continuously inject their antigens into the bloodstream and increase the risk of psoriasis[36,38,39]. Based on an *in vitro* study by Ruiz-Romeu *et al*[40] it was demonstrated that *S. pyogenes* could increase the activity of T cell lymphocytes by inducing Th17 responses in patients with guttate psoriasis. In general, according to previous studies as well as our study, on the one hand, infection with *S. pyogenes* increases the risk of psoriasis, and on the other hand, long-term treatment and prevention of its infection reduce the risk of psoriasis.

This study had several limitations including: low sample size, the use of English articles only, publication bias, presence of significant heterogeneity, and lack of subgroup analysis or sensitivity analyses to reduce heterogeneity. However, we

showed in this study that the rate of *S. pyogenes* infection in patients with psoriasis was about twice as high as that in healthy individuals. We also showed that infection with *S. pyogenes* could significantly increase the risk of psoriasis.

CONCLUSION

S. pyogenes can stimulate the skin through its enzymes, toxins, superantigens, and T-cell lymphocytes and increases the risk of psoriasis by dysregulating the immune response. Hence, *S. pyogenes* can be considered a risk factor for psoriasis, and prevention from *streptococcal* infection as well as effective treatment of *S. pyogenes* infections are among the best strategies to reduce the risk of psoriasis.

ARTICLE HIGHLIGHTS

Research background

Psoriasis is a multifactorial autoimmune disease, and it has been suggested that bacterial infection can contribute to the initiation or development of this disease.

Research motivation

We performed this study to discover the association between infection with *Streptococcus pyogenes* (*S. pyogenes*) as potential factors and risk of develop to psoriasis.

Research objectives

The objective of this study was to determine the impact of *S. pyogenes* infection and susceptibility to psoriasis using available case-control studies.

Research methods

We used a computer-assisted comprehensive literature search to obtain relevant case-control regarding to the possible connection between *S. pyogenes* infection and psoriasis. Finally, the impact of infection with *S. pyogenes* and susceptibility to psoriasis was measured using odds ratio (OR) with 95% confidence intervals (CIs).

Research results

The rate of infection with *S. pyogenes* in psoriatic patients *vs* healthy individuals was 33.4% and 16.2%, respectively. Furthermore, there is a significant association between *S. pyogenes* infection and development of psoriasis (OR: 6.58; 95%CI: 3.64-11.87; P = 0.001)

Research conclusions

Infection with *S. pyogenes* is a risk factor for susceptibility to psoriasis.

Research perspectives

Further long-term cohort studies are needed to investigate the relationship between *S. pyogenes* infection and psoriasis. Also, studies are needed to evaluate the clinical efficacy of the treatment of *S. pyogenes* infection in decreasing the number of psoriatic events.

ACKNOWLEDGEMENTS

We thank our colleagues from Mashhad University of Medical Sciences and Jiroft University of Medical Sciences.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 June 28; 9(3): 317-326

DOI: 10.13105/wjma.v9.i3.317

ISSN 2308-3840 (online)

META-ANALYSIS

Extraintestinal infection of *Listeria monocytogenes* and susceptibility to spontaneous abortion during pregnancy: A systematic review and meta-analysis

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Author contributions: The study was conceived by Keikha M; Keikha M and Karbalaei M analyzed the data; Yousefi A and Karbalaei M wrote the draft; Keikha M provided critical analysis and revision the draft; All authors read and approved the final manuscript.

Conflict-of-interest statement: There is no conflict of interest among the authors.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Listeria monocytogenes (*L. monocytogenes*) is one of the most important zoonotic bacteria that is transmitted to humans through infected animal products and is the cause of human listeriosis. Pregnant women and immunocompromised patients are more susceptible to the bacterium than healthy people. Recent studies have reported extensive evidence on the role of *L. monocytogenes* infection and the risk of spontaneous abortion.

AIM

To evaluate the possible connection with *L. monocytogenes* in the risk of spontaneous abortion in pregnancy.

METHODS

We conducted a systematic literature review using several databases to search the relevant case-control studies on the association between *L. monocytogenes* infection and spontaneous abortion. Finally, the impact of infection with *L. monocytogenes* and risk of spontaneous abortion was assessed *via* odds ratio at corresponding 95% confidence intervals.

RESULTS

In the present study, we evaluated the data of 4059 pregnant women who had a spontaneous abortion, and interestingly their colonization rate of *L. monocytogenes* was about 20.5%.

CONCLUSION

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Manuscript source: Unsolicited manuscript

Specialty type: Medicine, research and experimental

Country/Territory of origin: Iran

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 23, 2021
Peer-review started: April 23, 2021
First decision: June 7, 2021
Revised: June 13, 2021
Accepted: June 16, 2021
Article in press: June 16, 2021
Published online: June 28, 2021

P-Reviewer: Zhang LL
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Li JH

Therefore, based on statistical analysis, we found that there is a significant relationship between the infection with *L. monocytogenes* and spontaneous abortion.

Key Words: *Listeria monocytogenes*; Meta-analysis; Pregnancy; Spontaneous abortion; Women

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Core Tip: *Listeria monocytogenes* is one of the most common bacterial infections among developing countries. Acquisition of this bacterium during pregnancy is dangerous for the health of neonates. Our results suggested that *Listeria monocytogenes* can significantly increase the risk of spontaneous abortion during pregnancy.

Citation: Yousefi A, Karbalaei M, Keikha M. Extraintestinal infection of *Listeria monocytogenes* and susceptibility to spontaneous abortion during pregnancy: A systematic review and meta-analysis. *World J Meta-Anal* 2021; 9(3): 317-326
URL: https://www.wjgnet.com/2308-3840/full/v9/i3/317.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i3.317

INTRODUCTION

Spontaneous abortion is one of the biggest health challenges that has increased in recent decades, especially due to *in vitro* fertilization methods, such as *in vitro* fertilization, gamete intrafallopian transfer, and intracytoplasmic sperm injection[1]. About 1% of women suffer from recurrent miscarriage, so this phenomenon can be considered a serious threat to population decline[1,2]. The prevalence of spontaneous abortion in the world is reported to be 10%-20%, and many factors such as blood type, ectopic pregnancy, smoking, obesity, history of cesarean section, fertility treatment, and infectious pathogens are involved in its occurrence[3,4]. According to the literature, several infectious microorganisms such as *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Listeria monocytogenes* (*L. monocytogenes*), herpes simplex viruses, adenoviruses, human polyomaviruses, and cytomegalovirus play an important role in individual susceptibility to spontaneous abortion[5,6].

L. monocytogenes is a gram-positive, non-spore forming, facultative anaerobe, and motile bacterium that naturally resides in sources such as water, soil, vegetables, dairy products, and processed food[7]. These bacteria enter the human body through the fecal-oral route and can withstand harsh environmental conditions such as acidic pH, high salt concentration, and low temperature[8]. This bacterium can cross the intestinal barrier and enter various tissues through hematogenous dissemination, particularly the placenta-fetal unit and the cerebrospinal fluid[9,10]. Studies show that pregnant women are about 17 times more likely to be infected with *L. monocytogenes* than the general population[11]. Numerous studies have been performed on the effects of infection of *L. monocytogenes* and abortion in humans and animals[12]. The aim of this study was to investigate the effects of extraintestinal infection with this pathogen and susceptibility to spontaneous abortion. We estimated the frequency of *L. monocytogenes* colonization in the women who had a spontaneous miscarriage.

MATERIALS AND METHODS

Search strategy and study selection

Initially, a systematic search of global databases such as PubMed, Scopus, Google Scholar, and SID was conducted to collect all articles on the effects of *L. monocytogenes* infection on spontaneous abortion, regardless of publication date or language restrictions. Search terms were selected based on Medical Subject Headings browser including "*Listeria monocytogenes*," "Spontaneous abortion," "Pregnant women," and "Pregnancy." Articles published in Persian, Arabic, and English were the only articles

evaluated. After reviewing the titles and abstracts of eligible original articles (case-control studies, cross-sectional, and longitudinal studies), we selected those studies that were related to the effect of *L. monocytogenes* infection on spontaneous abortion. The process of searching and evaluating articles was done by two authors independently, and the discrepancies were examined by the third author. Duplicate articles, insufficient and vague information, case reports, and review articles were excluded from the study (Figure 1).

Data extraction and quality assessment

The required information such as first author, publication year, country, number of cases with at least one spontaneous abortion, number of *L. monocytogenes* strains, frequency of *hlyA* gene, frequency of serotypes, diagnostic methods, and the reference number were extracted from the eligible studies and are presented in Table 1. An important evaluation checklist of the Joanna Briggs Institute was used to evaluate the quality of the studies in the present meta-analysis.

Statistical analysis

The comprehensive meta-analysis software ver 2.0 (Biostat, Englewood, NJ, United States) was used for data pooling as well as statistical analysis of the extracted data. The frequency of *L. monocytogenes* infection in women with spontaneous abortion was measured as the incidence rate (event rate) with 95% confidence intervals (CIs) using cross-sectional and case-control studies. In addition, the frequency of the *hlyA* gene as well as common serotypes isolated from women with spontaneous abortion were also examined as the incidence rate. The effect of infection with this bacterium on the susceptibility to spontaneous abortion was assessed according to odds ratio with the 95%CIs using eligible case-control studies. Finally, the frequency of resistance of *L. monocytogenes* strains to different antibiotics was calculated and reported as the incidence rate and heterogeneity between studies was determined using I^2 index and Cochran's *Q*-test. In cases with high heterogeneity (I^2 > 25% and Cochran's *Q*-test *P* > 0.05), the analysis was done using the random-effects model. The publication bias was also determined using Egger's *P* value, Begg's *P* value, and asymmetry of funnel plots.

RESULTS

Characteristics of eligible studies

Of the 732 initial studies collected, 24 studies were eligible and entered in our meta-analysis (Figure 1)[13-36]. Of these, 17 studies were conducted in Iran, 2 studies in India, 2 studies in Iraq, 1 study in Bosnia and Herzegovina, 1 study in Nigeria, 1 study in Nagpur, and 1 study in Senegal. Overall, in selected studies *L. monocytogenes* (strains were isolated from urine, blood, placenta, vaginal swabs, and cervix swabs). In general, microbiological methods such as cold-enrichment, culture on PALCAM agar, culture on blood agar, as well as Gram-staining, oxidase, catalase, carbohydrates fermentation, methyl red/Voges-Proskauer, and motility at 25 °C were used to isolate and identify *L. monocytogenes* strains. In addition, the authors had used polymerase chain reaction technique (for genes such as *hlyA*, *InlA*, *InlB*, *actA*, *iap*, *plcA*, and *PrfA*) and serological tests on blood samples in their studies (Table 1).

Statistical analysis of spontaneous abortion

The prevalence of *L. monocytogenes* infection in women undergoing spontaneous abortion was approximately 20.5% (18.9-22.3 with 95%CIs; *P* = 0.001; I^2: 93.85; *Q*-value: 374.16; *P* = 0.001; Egger's *P* = 0.05; Begg's *P* = 0.01) was estimated (Figure 2).

The frequency of the *hlyA* gene in *L. monocytogenes* strains isolated from patients was estimated to be about 28.0% (21.0%-36.3% with 95%CI; *P*= 0.001; I^2: 87.34; *Q*-value: 94.84; *P* = 0.001; Begg's *P* = 0.03; Egger's *P* = 0.001). Also, abundance of serotypes 1/2a, 12/b, and 4b were measured to be about 38.7% (26.8%-52.2% with 95%CIs), 26.5% (13.1%-46.3% with 95%CIs), and 49.5% (32.8%-59.5% with 95%CIs), respectively.

Furthermore, it was revealed that the infection with *L. monocytogenes* significantly increases the risk of spontaneous abortion in pregnant women (odds ratio: 2.778; 2.130-3.623 with 95%CIs; *P* = 0.001; I^2: 76.30; *Q*-Value: 21.01; *P* = 0.001; Egger's *P* = 0.38; Begg's *P* = 0.51) (Figure 3).

Statistical analysis of antibiotic resistance

The current analysis showed that *L. monocytogenes* strains isolated from spontaneous

Table 1 Characteristics of included studies

Ref.	City	Number of patients		Number of positive culture for *L. monocytogenes*		Number of *hylA* positive strains	Frequency of serotypes	Diagnostic method
		Case	Control	Case	Control			
Aljicevićet *al*[13], 2005	Bosnia and Herzegovina	30	30	18	8	ND	ND	Serology
Kaur *et al*[14], 2007	India	61	ND	4	ND	3	ND	Convectional
Saeedi *et al*[15], 2009	Iran	118	99	9	3	ND	ND	IgG
Jamshidi *et al*[16], 2009	Iran	250	200	89	35	ND	ND	IgG
Tahery *et al*[17], 2009	Iran	102	102	82	20	ND	ND	IFA
Nazeri[18], 2011	Iran	512	ND	6	ND	ND	ND	PCR
Lotfollahi *et al*[19], 2011	Iran	100	ND	9	ND	ND	ND	Convectional
Jahangirisiskht *et al* [20], 2012	Iran	190	120	9	2	11/107	ND	Convectional
Jahangirsisakht *et al* [21], 2013	Iran	64	ND	7	ND	10.28	ND	PCR
Shindang *et al*[22], 2013	Nigeria	200	ND	14	ND	ND	4b (71%); 1/2a (28)	Convectional
Eslami *et al*[23], 2014	Iran	96	ND	16	ND	16	ND	PCR
Shoukat *et al*[24], 2014	India	141	ND	4	ND	4	ND	Convectional
Haghroosta *et al* [25], 2015	Iran	120	60	25	4	ND	ND	IFA
Pourkaveh *et al*[26], 2016	Iran	317	ND	54	ND	11		PCR
Bobade *et al*[27], 2016	Nagpur	113	ND	11	ND	11	4b (81%); 1/2b (18%)	PCR
Tajedini *et al*[28], 2017	Iran	58	ND	21	ND	ND	ND	IFA
Pour *et al*[29], 2018	Iran	123	ND	28	ND	28	1/2a (50%); 4b (35%)	PCR
Heidari *et al*[30], 2018	Iran	100	ND	4	ND	4	ND	Convectional
Al-dorri[31], 2018	Iraq	94	ND	11	ND	11	ND	PCR
Ohadi *et al*[32], 2019	Iran	96	ND	16	ND	16	1/2b (31%); 1/2a (25%); 4b (12.5%)	PCR
Mozaffari *et al*[33], 2019	Iran	130	100	35	11	ND	ND	IFA
Zahirnia *et al*[34], 2019	Iran	124	76	31	28	3	ND	PCR
Fall *et al*[35], 2020	Senegal	43	ND	2	ND	ND	ND	PCR
Al-Mayahi and Jaber[36], 2020	Iraq	90	ND	15	ND	15	ND	PCR

L. monocytogenes: *Listeria monocytogenes*; PCR: Polymerase chain reaction; ND: Not detected; IFA: Increased femoral anteversion; IgG: Immunoglobulin G.

abortion cases were resistant to various antibiotics. The resistance to different antibiotics was as follows: ampicillin 31.8% (25.5-38.9 with 95%CIs; I^2: 92.96; *Q*-Value: 56.84; *P* = 0.001), penicillin G 56.8% (51.4-62.0 with 95%CIs; I^2: 94.64; Q-Value: 74.67; *P* = 0.001), cotrimoxazole 33.0% (24.5-42.8 with 95%CIs; I^2: 0.00; *Q*-Value: 0.00; *P* = 1.00), cephalothin 50.0% (40.3-59.7 with 95%CIs; I^2: 0.00; *Q*-Value: 0.00; *P* = 1.00), tetracycline 29.2% (23.2-36.1 with 95%CIs; I^2: 91.75; *Q*-Value: 36.37; *P* = 0.001), erythromycin 15.0%

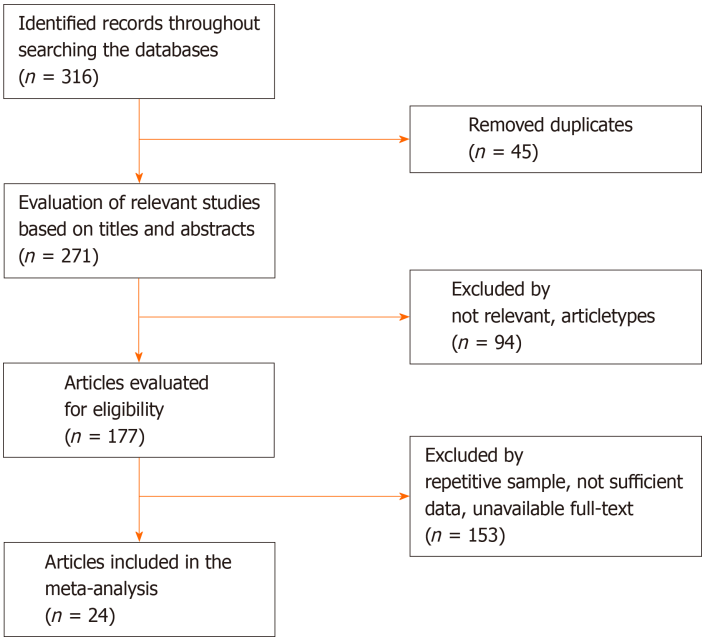


Figure 1 Flowchart of search strategy and study selection.

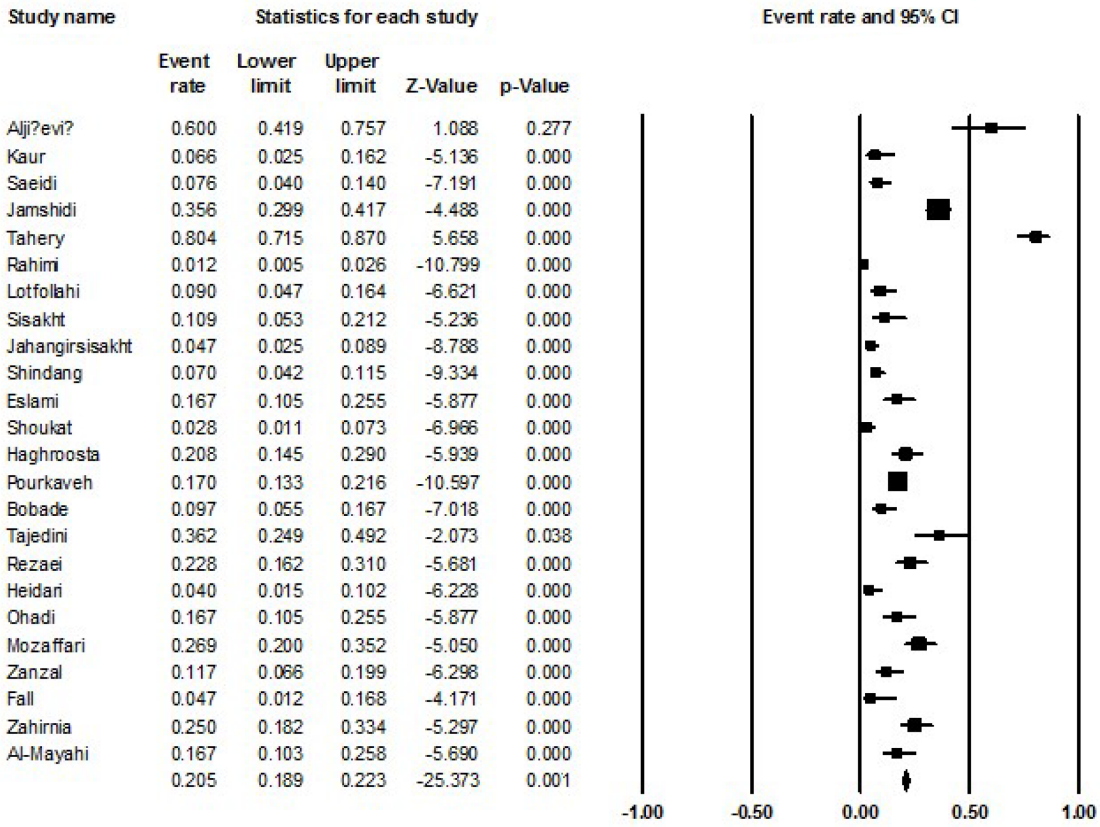


Figure 2 Forest plot for frequency of infection with *Listeria monocytogenes* among spontaneous abortion cases.

(9.5-22.8 with 95%CIs; I^2: 91.96; *Q*-Value: 37.31 ; *P* = 0.001), cefotaxime 77.0% (70.7-82.3 with 95%CIs; I^2: 0.00; *Q*-Value: 0.00; *P* = 1.00), chloramphenicol 11.0% (7.4-16.1 with 95 % CIs; I^2: 0.00; *Q*-Value: 0.00; *P* = 1.00), trimethoprim 38.3% (30.5-46.8 with 95%CIs; I^2: 95.47; *Q*-Value: 66.26; *P* = 0.01), ciprofloxacin 11.1% (6.8-17.7 with 95%CIs; I^2: 82.1; *Q*-Value: 11.18; *P* = 0.04), gentamycin 28.0% (20.0-37.6 with 95%CIs; I^2: 0.00; *Q*-Value: 0.00; *P* = 1.00), and streptomycin 11.0% (7.4-16.1 with 95%CIs; I^2: 0.00; *Q*-Value: 000; *P* = 1.00). No resistance has been reported to the three antibiotics amikacin, meropenem,

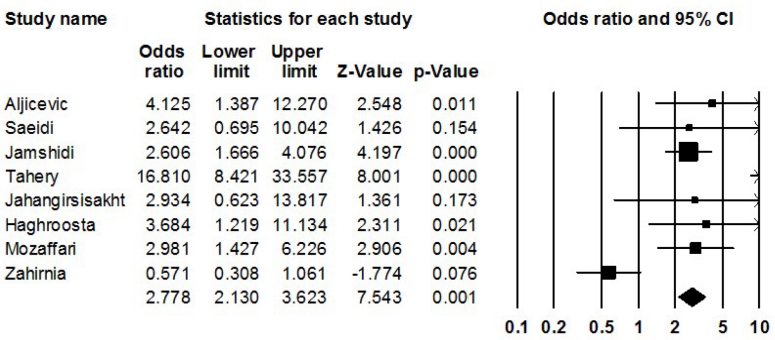


Figure 3 Forest plot for relationship between *Listeria monocytogenes* infection and susceptibility to spontaneous abortion.

and norfloxacin.

DISCUSSION

Listeriosis is one of the most well-known foodborne diseases that is transmitted to humans through the consumption of contaminated animal products. Several outbreaks of listeriosis have been reported worldwide, with children being the main victims[37]. According to studies, the prevalence of listeriosis *per* 100000 people in children, the elderly, and pregnant women is estimated at 3.4, 10.0, and 12.0, respectively. Pregnant women can pass listeriosis to their baby in the womb or at birth[38]. Latent listeriosis in pregnant women can have fatal effects on the fetus such as granulomatosis infantiseptica, spontaneous abortion, stillbirth, premature birth, and meningitis[39]. According to the Centers for Disease Control and Prevention, about 14% of human listeriosis occurs in pregnant women. Therefore, the diagnosis of listeriosis during pregnancy is essential, especially in developing areas (*Listeria* spp. are more likely to be contaminating food in these areas)[40].

Based on the current analysis, it was shown that the rate of colonization with *L. monocytogenes* in women who had spontaneous abortions was about 20.5%, although in some studies, *L. ivanovii* and *L. seeligeri* were the cause of infection and abortion. For the first time, in the current meta-analysis, we evaluated the association of colonization with *L. monocytogenes* in pregnant women with spontaneous abortion, and most of the studies considered in the current analysis were related to Iran. Iran as a developing country has a high rate of *L. monocytogenes* infection, which in turn is related to working conditions, lifestyle, and geographical location. In another meta-analysis study from Iran, the rate of colonization with *L. monocytogenes* in humans, animals, and food products was estimated at 10%, 7%, and 4%, respectively[41].

Cell-mediated immunity is somewhat suppressed during pregnancy, while humoral immunity is as active as ever. Therefore, pregnancy increases the risk of infection with facultative intracellular bacteria such as *L. monocytogenes*. Meanwhile, the human placenta also provides a protective niche for the growth and proliferation of this bacterium, which in turn increases the risk of spontaneous abortion[42]. We found that infection with *L. monocytogenes* can significantly increase the risk of miscarriage during pregnancy (odds ratio: 2.7; 2.1-3.6 with 95%CI; $P = 0.01$).

So far, four lineages have been identified from *L. monocytogenes* strains, and it should be noted that lineages I (serotypes 1/2b and 4b) and II (1/2a) are responsible for most human infections. In this study, the frequencies of serotypes 1/2a, 1/2b, and 4b were reported as 38.7%, 26.5%, and 49.5%, respectively, which were similar to the distribution of serotypes in other human listeriosis infections[27,29]. In pathogenic strains, numerous virulence factors are encoded by pathogenicity island 1. The *hlyA* gene encodes hemolysin (listeriolysin-O), which is one of the most important virulence factors of *L. monocytogenes*[43]. According to our analysis, only 28% of strains isolated from women with spontaneous miscarriage had this gene, indicating the low diagnostic value of this gene for diagnosis of *L. monocytogenes* infection in pregnant women.

Based on the evidence, β-lactam antibiotics alone or in combination with aminoglycosides as well as cotrimoxazole are recommended treatment options for severe listeriosis infections. Although, nowadays, the resistance of *Listeria* spp. to β-lactams and aminoglycosides is increasing[44,45]. Some *L. monocytogenes* strains are also

resistant to other antibiotics such as fluoroquinolones, macrolides, and tetracyclines, and the emergence and spread of the drug-resistant strains in pregnant women can be life-threatening for both mother and child[44]. Our results showed that the antibiotic resistance of *L. monocytogenes* strains isolated from the women with spontaneous abortion was high. The highest resistance was related to cefotaxime (77%), and the lowest was related to chloramphenicol and streptomycin (both 11%); all strains were sensitive to norfloxacin, meropenem, and amikacin. In a recent meta-analysis study by Khademi and Sahebkar[44], resistance of *L. monocytogenes* strains isolated from Iranian patients to penicillin, ampicillin, and gentamicin was reported to be 56.8%, 29.5%, and 32.4%, respectively, which was close to the results of our study[44]. Quentin *et al*[46] also identified a multi-resistant strain of *L. monocytogenes* in a septic abortion[46]. It is important to note that increasing drug resistance of *L. monocytogenes* strains to β-lactams and aminoglycosides in the coming years will lead to the ineffectiveness of these antibiotics in the treatment of human listeriosis.

Our meta-analysis has several limitations as following: (1) The population sample size was low; (2) Heterogeneity was significant; (3) There is a slight publication bias; and (4) We included only available articles in Persian, Arabic, and English. Overall, our results showed that there is a significant relationship between *L. monocytogenes* infection and the increased risk of miscarriage during pregnancy. The results of the present analysis also showed that drug resistance is increasing among *L. monocytogenes* strains, which needs to be re-evaluate. If necessary, treatment guidelines should be updated to reduce the incidence of human listeriosis.

CONCLUSION

Overall, regarding the importance of microorganisms such as *Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis, L. monocytogenes*, herpes simplex viruses, adenoviruses, human polyomaviruses, and cytomegalovirus in the induction of spontaneous abortion, it is important that all aspects of these pathogens, such as diagnosis, treatment, control, and vaccination, be considered by all researchers around the world.

ARTICLE HIGHLIGHTS

Research background

Spontaneous abortion is one of the most important concerning issues in pregnant women, and it has been suggested that *Listeria monocytogenes* (*L. monocytogenes*) infection can play a key role in pathogenesis of this disease.

Research motivation

We conducted the present study to estimate the risk of infection with *L. monocytogenes* in the development of spontaneous abortion during pregnancy.

Research objectives

The aim of this study was evaluation of the probable connection between infection with *L. monocytogenes* and risk of spontaneous abortion in pregnancy.

Research methods

We conducted a systematic literature review using several databases to search the relevant case-control studies on the association between *L. monocytogenes* infection and spontaneous abortion. Finally, the impact of infection with *L. monocytogenes* and risk of spontaneous abortion was assessed *via* odds ratio at corresponding 95% confidence intervals.

Research results

The frequency of *L. monocytogenes* infection was significantly increased in pregnant women with spontaneous abortion in comparison with healthy subjects. There is significant association between infection with *L. monocytogenes* and development of spontaneous abortion in pregnant women (odds ratio: 2.778; 2.130-3.623 with 95% confidence interval).

Research conclusions

Our results suggested the infection with *L. monocytogenes* is a marker for prediction of the risk of development of spontaneous abortion during pregnancy.

Research perspectives

Regarding the importance of *L. monocytogenes* in the initiation and development of spontaneous abortion, it is important that all aspects of this pathogen, such as diagnosis, treatment, control, and vaccination, be considered by all researchers around the world.

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ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 August 28; 9(4): 327-404



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World Journal of Meta-Analysis

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World Journal of Meta-Analysis

Bimonthly Volume 9 Number 4 August 28, 2021

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INDEXING/ABSTRACTING

The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Saurabh Chandan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2308-3840/editorialboard.htm

PUBLICATION DATE
August 28, 2021

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 August 28; 9(4): 327-332

DOI: 10.13105/wjma.v9.i4.327

ISSN 2308-3840 (online)

EDITORIAL

Watch and wait in locally advance rectal cancer: Past, present and future

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Author contributions: Alvarez-Aguilera M and Jimenez-Rodriguez M are responsible for the conception, design and writing of this work; Both authors have contributed equally to this work.

Conflict-of-interest statement: The authors declare that they have nothing to disclose.

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Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: Spain

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Abstract

In rectal cancer, a complete pathological response after neoadjuvant therapy means better rates survival and better rates of local recurrence. Nevertheless, these patients suffer from complications following surgery such as low anterior resection syndrome, sexual dysfunction or colostomy for the rest of their lives. Due to this, several groups are working in an organ preservation strategy when a clinical response is diagnosed. This strategy is known as watch and wait. In this editorial, we review the past, present and future perspectives for this conservative management.

Key Words: Rectal cancer; Watch and wait; Neoadjuvant treatment; Organ preservation; Complete response

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Core Tip: Organ preservation for rectal cancer after neoadjuvant therapy should be considered for selected patients with clinical complete response after neoadjuvant therapy.

Citation: Alvarez-Aguilera M, Jimenez-Rodriguez RM. Watch and wait in locally advance rectal cancer: Past, present and future. *World J Meta-Anal* 2021; 9(4): 327-332

URL: https://www.wjgnet.com/2308-3840/full/v9/i4/327.htm

DOI: https://dx.doi.org/10.13105/wjma.v9.i4.327

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 30, 2021
Peer-review started: April 30, 2021
First decision: July 4, 2021
Revised: July 27, 2021
Accepted: August 17, 2021
Article in press: August 17, 2021
Published online: August 28, 2021

P-Reviewer: Ergun Y
S-Editor: Liu M
L-Editor: Filipodia
P-Editor: Li JH

INTRODUCTION

The treatment for rectal cancer has changed in the last years due to technical advances: From the surgical technique, to stage and to pre and postop treatments. Nowadays, the standard treatment includes neoadjuvant therapy followed by total or subtotal mesorectal excision and adjuvant therapy[1] achieving excellent local control rates. Nevertheless, a third of the patients diagnosed with rectal cancer died due to distant metastasis[2].

Moreover, this multimodal treatment still has complications, including those coming from surgery: Infections, permanent ostomies, toxicity from chemo and radiotherapy, neurotoxicity or anterior resection syndrome and in some cases even death[3-5].

All these adverse effects cause a decrease in the quality of life of our patients. Due to this, current trend is to tailor the treatment regarding the tumor, its size, location stage and molecular characteristics as well as the own patient.

An example of this tailored treatment is the PROSPECT trial[6], a phase 2/3 clinical trial proposing to suppress systematic preoperative chemoradiotherapy. The authors describe the administration of 6 cycles of FOLFOX as neoadjuvant therapy followed by re-stage of the tumor and selectively use of radio and chemotherapy regarding the response of the tumor.

Despite the different schemas the final objective of all these preoperative treatments is maximize the number of tumor complete responses because these patients with pathological complete response (pCR) will develop a local recurrence rate of 1% and 95% 5-year survival[7-10]. In addition to this, this patients without viable tumor in the specimen could benefit from a non-surgical management thus if we increase the rate in complete responses we will increase the number of patients which could benefit from a non-operative management.

This non-surgical management or watch and wait is not new. Traditionally it has been attempted in frail and old patients with high risk for surgery and with response to the neoadjuvant therapy. We could name this strategy as a "casual watch and wait" or "watch and wait by necessity".

Recently this strategy has been published as intentional and not casual watch and wait. Habr-Gama *et al*[11] described in their work in 2004 their results after treating with neoadjuvant therapy, 265 patients with rectal cancer. These patients were reassessed 8 wk after the end of the neoadjuvant therapy and those with complete clinical response entered into a watch and wait policy. Those patients without complete clinical response underwent surgery. Disease-free survival rates were 100% and 92% after 57 mo. In this work, authors reported 2 local regrowths who underwent salvage surgery and stayed alive and disease-free at the end of the study.

Habr-Gama *et al*[11] concluded that in association with a careful follow-up, watch and wait for rectal cancer is safe and feasible.

More recent studies show pool analysis cases suggesting that watch and wait (WW) is feasible with similar survival rates to the traditional surgery and regrowth rates lower than 20%[12].

There is a difference between complete clinical response (cCR) and pCR. The first one is the absence of tumor after neoadjuvant therapy and the second one is the absence of viable tumor cells in the specimen after surgery. And sometimes these 2 options do not overlap. Current test cannot distinguish between tumor cells and fibrosis. In fact, up to 38% patients with an incomplete clinical response show after surgery pathological complete response[9]. This is a difficult situation for a patient and for a surgeon who have to choose between planning one or other approach.

There are different ways of increasing clinical complete response with the aim of increasing the number of patients who can benefit from a watch and wait strategy. One strategy is by optimizing preoperative radiotherapy. There are 2 ways of administering radiotherapy: Short course (25 Gy in 5 fractions) or long course (2 Gy fractions for in 2 Gy sessions for a total of 40 Gy to 50.4 Gy). Two prospective randomized trials [13,14] analyzed differences between these 2 modalities. The findings suggest there are no differences regarding local recurrences, survival or toxicity but there are differences regarding the rate of complete response in the specimen (0.7% in the short course modality against 16% in patients receiving long course radiotherapy). Other studies such as the one published by Bujko *et al*[13] and Cummings *et al*[15] studied the results of the short course in elderly population. These studies have a very small population, 30 and 20 cases respectively, and both studies concluded that WW is feasible after short course radiotherapy in elderly patients. Other studies such as the RAPIDO trial [16] randomized all patients to chemoradiotherapy (CRT) followed by surgery and optional adjuvant therapy or to total neoadjuvant therapy including short course

radiotherapy, 6 cycles of CapeOx and followed by surgery will show more information about the role of short course radiotherapy in WW.

As far as increasing tumor radio sensibility, the German study CAO/ARO/AIO-04 [17] demonstrated the benefit of adding oxaliplatin to CRT to maximize the outcomes of neoadjuvant therapy in terms of complete pathological response (17% *vs* 13%). This finding was consolidated after the follow-up with a 76% *vs* 71% 3-year disease-free survival (DFS). Nowadays the RAPIDO trial[16] has shown differences in toxicity in the experimental group (84%) but not in postoperative complications.

Another aspect is increasing waiting time after the end of neoadjuvant therapy. Tumor response to radiotherapy depends on the waiting time and sometimes a complete response could be delayed months. Although tumoral damage is produced during radiotherapy the lysis of the cells occurs days and even weeks after the end of the treatment. Surgery delay could increase the rate of pathological complete response from 0%, right after finishing CRT to 11% when the interval is 11 wk[15]. From that moment the rate of pathological complete response does not increase. Other studies also showed that an increase in complete response rates means a decrease in complication and readmission rates after surgery and similar survival rates. The TiMiSNAR trial[18] included 300 patients divided in 2 groups: Those receiving surgery 8 wk after surgery and those who underwent surgery 12 wk after CRT to determine when we should diagnose a pCR without affecting oncological outcomes.

Another strategy is adding chemotherapy to neoadjuvant therapy like induction or consolidation. There are studies that analyzed the result of induction or consolidation therapies in patients that also receive the classical schema: CRT and surgery. Consolidation therapy[19] has been associated to less toxicity, higher rate of completion and higher rates of pCR (25% *vs* 17%). Current evidence supports consolidation therapy as more favorable to increase the number of pCR and thus, WW. Nevertheless, we are still waiting the results coming from studies like the OPRA trial[20] where patients are randomized to induction or consolidation and surgery or WW.

All these strategies increase the rate of pCR and as consequence higher rates of WW. But once we decide we can offer a WW strategy, what should we do?

The first step is to determine the precise stage. Stage 2 and 3 tumors will benefit from neoadjuvant treatment, will possibly respond and will be candidates for a WW strategy. However, stage 1 tumors can also be included. This stage should be done with rectal exam, endoscopy and magnetic resonance imaging (MRI). Incomplete or complete response will be considered regarding our findings (Table 1).

Patient should be trustable and involved with the follow-up, to detect regrowths and perform surgery if needed. Follow-up should be performed as described in Table 2.

A group of patients will have more options of benefit from a WW strategy, for example those with more distal or smaller tumors requiring ultralow anastomosis or abdominoperineal resections. Bigger and circumferential tumors usually develop concentric scars inside the rectum obstructing the assessment of the tumor response. These patients could not be the ideal candidates for a WW program. Those with a higher risk of tumor progression might not be good candidates for this non-surgical management either. Smith *et al*[5] published a higher risk of distant metastasis in those patients who developed a regrowth (36% *vs* 1%, $P < 0.001$). This author declares the higher rate in the regrowth group could be due to delays in surgery or to more aggressive tumors.

We should begin with neoadjuvant treatment including chemo and radiotherapy and assess after 8-12 wk. At this point, there are different options:

cCR: scar, telangiectasia, and findings in the MRI suggesting cCR. We could suggest follow-up.

Incomplete clinical response: surgery.

Nearly complete clinical complete response: A follow-up could be suggested every 6-8 wk if the tumor still responds. If there is a regrowth, we should recommend surgery.

These recommendations are likely to be modified in the next few months with the information obtained from the different ongoing studies described previously, such as the OPRA[20] trial, that aim to assess the results of consolidation chemotherapy.

As we mentioned before, a certain group of patients might experience tumor regrowth during WW follow-up. A variety of studies claim that this scenario is more likely to show up in the first 2 years after neoadjuvant treatment rather than later on [5]. After carefully analyzing the graphical representation of these regrowth patterns, only a few patients are at risk of suffering this regrowth. Nevertheless, it is certainly hard to determine who will experience this, when it will happen, and which follow-up method may be the most effective one (DNAc, biopsies, imaging, *etc.*).

Table 1 Clinical response criteria regarding findings in rectal exam, endoscopy and magnetic resonance imaging described in OPRA trial[20]

	Clinical complete response	Almost complete response	Incomplete response
Endoscopy	Plain, white scar	Irregular mucosal layer	Visible tumor
	Telangiectasia	Minimal nodules or rugosity in the mucosal layer	
	Absence of ulceration	Superficial ulceration	
	Absence of nodules	Light and persistent scar erythema	
Digital rectal exam	Normal	Smooth induration or light mucosal layer alterations	Palpable nodules
MRI T2W	Dark signal in T2 without intermedial signal and no visible adenopathies	Dark signal in T2 with an intermedial signal	More intermediate-dark signal in T2
		And/or	No scar in T2
		Partial regression of the adenopathies	And/or
			No adenopathies regression
MRI DW	No visible tumor with B800-B1000 signal	Significant regression of the signal in B800-B1000	Insignificant signal regression in B800-B1000
		And/or	And/or
	And/or	Minimal/ residual signal in ADC map	Clear low signal in ADC map
	Absent/low signal in ADC map		
	A linear uniform signal in the tumor wall is a non-pathological sign		

ADC: Apparent diffusion coefficient; MRI: Magnetic resonance imaging.

Table 2 Recommendations for follow-up for patients under a watch and wait strategy regarding OPRA trial[20]

Follow-up in mo	3-6	9-12	15-18	21-24	30	36	42	48	54	60
PE	√	√	√	√	√	√	√	√	√	√
Endoscopy	√	√	√	√	√	√	√	√	√	√
MRI	√	√	√	√	√	√	√	√	√	√
CT CAP	√	√	√	√	√	√	√	√	√	√
TM	√	√	√	√	√	√	√	√	√	√

CT CAP: Computed tomography chest, abdomen and pelvis; MRI: Magnetic resonance imaging; PE: Positron emission; TM: Tumor markers.

Several authors have published failure rates after a WW trial reporting a wide range of results, from 3% described by Habr-Gama *et al*[11] in his first study up to a 30% in other publications[21], clearly higher numbers when compared to the Brazilian group. It might be due to the exclusion of the first 12 mo of follow-up in the Brazilian group, and consequently the first 12-mo regrowth rate.

More recent studies show locoregional recurrence rate of about a 19%[5,20] as the closest to the real number. This recurrence might occur deep into the mucosal layer and therefore could be difficult to detect before the sphincteric complex is affected. However, in the presence of this scenario, savage surgery can be offered to the patient achieving a similar survival rate as to an initial surgery without a WW first step.

CONCLUSION

Patients with rectal cancer who undergo neoadjuvant therapy could present a complete clinical response. When this happens, these patients might benefit from a

non-surgical strategy associated with an exhaustive follow-up as long as they are aware of the implications of this pathway. In the event of a tumor regrowth, savage surgery can be offered to them with similar oncological results. Nonetheless, future investigations are in order to elucidate the most proper candidates and follow-up methods for this treatment alternative.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 August 28; 9(4): 333-341

DOI: 10.13105/wjma.v9.i4.333

ISSN 2308-3840 (online)

MINIREVIEWS

Fate of root shell after pontic/socket shield techniques, is it better to extract the whole tooth?

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Author contributions: Agrawal AA has solely contributed to this paper in all aspects.

Conflict-of-interest statement: Agrawal AA has nothing to disclose.

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Manuscript source: Invited manuscript

Specialty type: Dentistry, oral surgery and medicine

Country/Territory of origin: India

Peer-review report's scientific quality classification

Grade A (Excellent): 0

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Abstract

A series of changes occur in the remaining alveolar process after whole tooth extraction. The basic question is, why do the bony walls (especially the labial/buccal) get resorbed immediately after the tooth is removed? This could be because, with cementum of the concerned tooth and its periodontal ligament, the supporting bundle bone is dependent on the presence of the tooth. This loss can be compensated using numerous techniques, such as socket grafting using various biomaterials to preserve the alveolar bone and buccal grafting with guided tissue regeneration to increase the thickness of buccal bone or placement of implant immediately. However, none of these techniques prevent the modelling of the alveolar bone post-extraction. Few studies have demonstrated that preservation of the roots in the alveolar process maintains the bone volume and facilitates vertical bone growth. A histological study in animals and humans has shown that the retained root shell does not pose any interference in the osseointegration of the implant (if placed simultaneously). Although various names have been proposed to describe the concept of retaining full or part of the root to prevent the resorption of the ridge, socket-shield and pontic-shield are the two most commonly used terms worldwide. The extraction of the whole tooth might be the choice of therapy when socket-shield or pontic-shield is not possible due to anatomical variations, infections, or lack of clinical expertise. Irrespective of the size, when a whole root or a root fragment (is left *in situ*), it is the dentist's ethical duty to advise/inform the patient and ensure repeated clinical and radiographic follow-up. The present study aimed to highlight the current status of these techniques, their benefits, and possible complications and address whether the paradigm of the teeth extraction methods should be altered.

Key Words: Socket shield technique; Pontic shield technique; Alveolar resorption; Tooth root; Tooth extraction; Ridge preservation; Alveolar resorption

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Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: March 27, 2021
Peer-review started: March 27, 2021
First decision: June 15, 2021
Revised: July 1, 2021
Accepted: September 1, 2021
Article in press: September 1, 2021
Published online: August 28, 2021

P-Reviewer: Arisan V, Pan J
S-Editor: Liu M
L-Editor: A
P-Editor: Li JH

Core Tip: The phrase 'prevention is better than cure' holds very true when it comes to alveolar ridge resorption. A significant bone is lost within 3 mo after extraction of the whole tooth. Socket/pontic shield techniques can preserve the alveolar bone and prevent a lot of surgical and economic burden to the patient in restoring what could have been saved in a very economical and natural way. Although these procedures are technique sensitive, clinicians must expertise in these techniques, since preserving what can be preserved is not only scientifically desirable but also ethically advisable.

Citation: Agrawal AA. Fate of root shell after pontic/socket shield techniques, is it better to extract the whole tooth? *World J Meta-Anal* 2021; 9(4): 333-341
URL: https://www.wjgnet.com/2308-3840/full/v9/i4/333.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i4.333

INTRODUCTION

Subsequent to whole tooth extraction, a series of changes occur in the remaining alveolar process. This may prevent or pose difficulty in implant installation at a prosthetically driven position. Also, there is an increasing demand for functional restoration and its aesthetic aspect. This emphasizes the importance of retaining sufficient alveolar ridge volume to deliver a functionally and aesthetically acceptable implant-supported prosthesis.

The basic question is, why do the bony walls (especially the labial/buccal) get resorbed rapidly after the tooth is removed? The answer is that with the cementum of the concerned tooth and its periodontal ligament (PDL), the supporting bundle bone is dependent on the presence of the tooth. The structure of the buccal bony wall of anterior teeth also has the same structure, and it is also tooth-dependent[1]. Interestingly, the bundle bone has the potential to exist in a thinner dimension compared to the alveolar/basal bone because of the PDL that provides the functional, nutritional, and cellular source existence and maintenance.

The majority of the dimensional changes that occur as a part of socket healing are primarily observed in the first 3 mo after extraction. However, the reorganization of the alveolar ridge can continue for almost a year[2], indicating a greater degree of bone modelling in the first 3 mo, followed by remodelling (Figure 1). However, the rate and pattern of socket healing could be attributed to the biological differences among individuals, the size of the socket under consideration, the prominence of root in the arch, and the degree of surgical trauma induced during extraction. In addition, the reflection of mucoperiosteal flap/full-thickness flap might lead to bone resorption of the thin buccal bone walls[3-5]. However, various human clinical and animal studies could not conclude that extraction without reflection of full-thickness flap prevents the resorption of alveolar bone/crest. The studies highlighted that the extraction procedure induces significant surgical trauma that exceeds the effect (if any) of full-thickness flap reflection[6-9]. Since there is more bundle bone at the crest of the buccal bone than the lingual, bone loss is pronounced in the buccal wall.

To compensate for this loss, there are numerous techniques described in the literature. Socket grafting (with various biomaterials) to preserve the alveolar bone (Figure 2C), buccal grafting with guided tissue regeneration (GTR) to increase the thickness of the buccal bone (Figure 2D), or placement of immediate implant (Figure 2E). However, none of these techniques truly prevent the modelling of the alveolar bone post-extraction[10-12]. This could be ascribed to significant alterations after tooth extraction due to the loss of PDL and subsequent trauma to the buccal bone. Thus, it could be hypothesized that root retention (vital/pulpless) may avoid tissue alterations that usually occur after whole tooth extraction. Few studies have demonstrated that preservation of the roots in the alveolar process maintains the existing bone volume and helps vertical bone growth[13]. Eventually, instead of retaining the whole root, retaining only a part of the root in contact with the buccal bone plate could be acceptable as only the buccal bone site is at a major risk of resorption (Figure 2F). These methods, termed as 'socket shield' or 'pontic-shield' techniques, were examined in an investigation in beagle dogs. The histological results of this study revealed that there was no bone modelling observed on the buccal wall, indicating that no resorption occurred[14]. In this study, we described the pros and

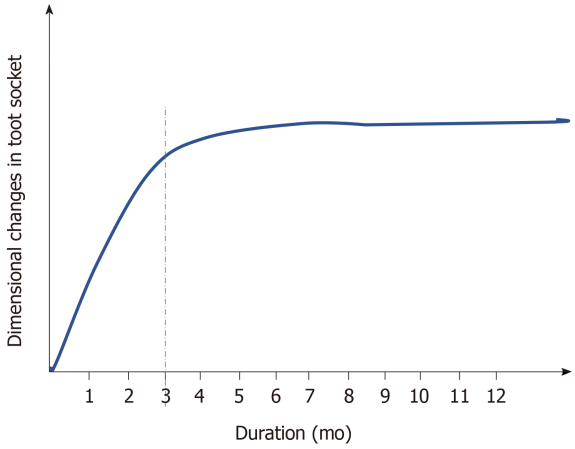


Figure 1 Dimensional changes as a part socket healing. This graph shows that after tooth extraction greater degree of bone modelling occurs in first 3-mo and the remodeling continue later on for a year and beyond.

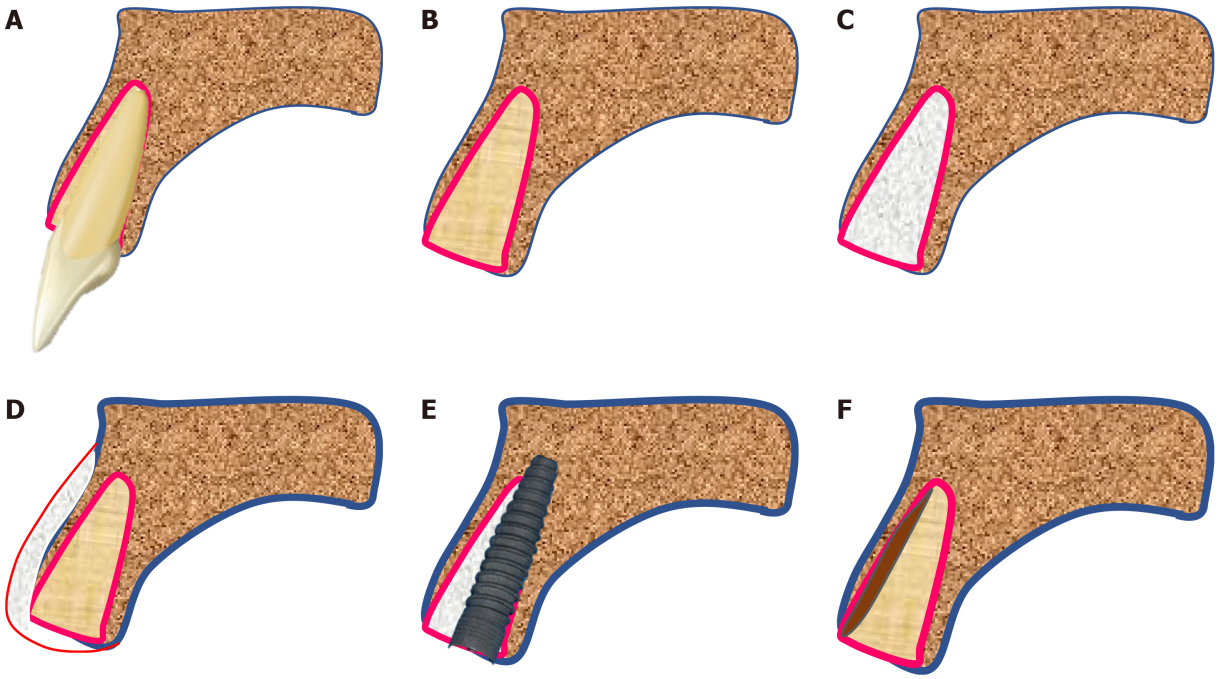


Figure 2 Techniques to compensate for the loss of socket wall post extraction. A: Tooth in socket; B: Empty socket after the tooth is extracted; C: Socket grafting (with lots of different biomaterials) to preserve the alveolar bone; D: Buccal bone grafting with GTR (guided tissue regeneration) to increase the thickness of buccal bone; E: Placement of immediate implant (with/without grafting the jumping space); F: Retaining only a part of root in contact with the buccal bone plate (socket-shield).

cons of leaving the root fragment behind intentionally. Although it might be rational to adopt this technique, whether extracting the whole tooth would is beneficial or leaving a part of the root is justified is yet to be investigated.

NOMENCLATURES AND TECHNICAL TIPS

During extraction, root pieces/root fragments might get retained inadvertently, which is the most common finding on routine radiographs[15]. Partial extraction therapies (PET) is a wide term that encompasses the different types and modifications of complete and partial root fragment retention. It is one of the earliest attempts of PET, wherein the submergence' technique has been successfully demonstrated for the preservation of alveolar ridge post-extraction, as well as the development of pontic sites. The crown of the tooth is sectioned at the bone crest, and the coronal aspect of the remaining root is hollowed out to mimic the future ovate pontic. Primary soft

tissue closure is recommended to encourage healing by primary intention. In a human study, Garver and Fenster[16] demonstrated that the resorption of the alveolar bone is reduced significantly when the root is retained in the alveolar process. Although the concept might be traditional, root submergence is still considered an advantageous method for the development of a pontic-site in clinical practice, wherein the majority of the treatments involve implant prosthesis[17]. Scheuber *et al*[18] published a technique to preserve the alveolar ridge following posttraumatic ankylosis and external root resorption by retaining the de-crowned root parts. Davarpanah *et al*[19] conducted a case series study of unconventional implant treatment and demonstrated that immediate implants in direct contact with ankylosed teeth fragments were successfully preserved without any abnormalities over 2 years.

The socket-shield technique introduced by Hürzeler *et al*[14] involves the facial/buccal root fragment alone to retain the resorption of thin bundle bones. The tooth in concern is sectioned horizontally 1 mm above the bone crest (Figure 3B) and then sectioned longitudinally in facial and palatal halves (Figure 3C). Next, the palatal section was extracted (Figure 3D), and the facial root section is concaved with a long shank dental bur (Figure 3E). It also involves immediate placement of dental implant palatal to the retained root fragment. The jumping space, if any, can be grafted as possible (Figure 3F).

The widespread clinical use of the root-membrane technique started after the outcomes of the first longitudinal study published by Siormpas *et al*[20]. The study claimed that root membrane is an appropriate term as it focuses on the retention of root fragment in the form of a membrane.

A modification of socket-shield technique is termed the pontic shield technique[21]. The surgical procedure is the same as a socket shield, with the only difference being that an immediate implant is not placed. It facilitates space-filling with maximum bone, and then the implant may or may not be placed. Mitsias *et al*[22] advocated that root-membrane techniques, such as socket shield and others, could be termed as 'PDL-mediated ridge preservation for immediate implant placement'. In addition to the preservation of alveolar bone or buccal bone, the proximal socket-shield has been used to preserve the interdental papillae. It is useful when two or more adjacent implants are planned.

TECHNICAL ASPECTS

The partial extraction therapies, socket-shield or pontic-shield or their various modifications, are technique-sensitive. The procedure is associated with the risk of displacement of the retained root fragment or the buccal lamellar bone. In either of these different techniques, no consensus has been achieved with respect to the height or thickness of the root fragment. Glocker *et al*[23] advocated keeping the root fragment at the same level of the buccal alveolar ridge, which prevents the risk of fracture of the root fragment. On the other hand, Mitsias *et al*[22] preferred the root fragment to be at least 1 mm higher than the buccal crest, which would retain more PDL fibers and support more soft tissue at the crest. In an animal study, Tan *et al*[24] demonstrated that the degree of bone resorption is not affected by the height of the root fragment. However, it was positively correlated when the thickness of the root fragment was between 0.5 mm and 1.5 mm, and the bone resorption may decrease significantly. Extremely thick root fragment is stable but occupies more space leaving less for the implant. On the other hand, an overly reduced shield is unstable. Gluckman *et al*[25] recommended reducing the thickness of the retained fragment to approximately half its thickness from the root canal to the tooth's labial limit.

HISTOLOGICAL EVIDENCE OF FATE OF THE RETAINED ROOT FRAGMENT

In a 4-mo histological examination study, O'Neal *et al*[26] submerged 16 endodontically-treated roots. More than 50% of the sectioned surface was covered with bone in about 62.5% of root specimens, and also, complete bone coverage was identified on the cut surfaces in the 2-mo specimen. Guyer[27] submerged vital roots in humans and discovered that the two roots displayed radiographically normal conditions, and the alveolar ridge dimensions were maintained clinically for 27 mo. Plata *et al*[28] conducted a 12-wk histological evaluation of 12 vital submerged roots and reported

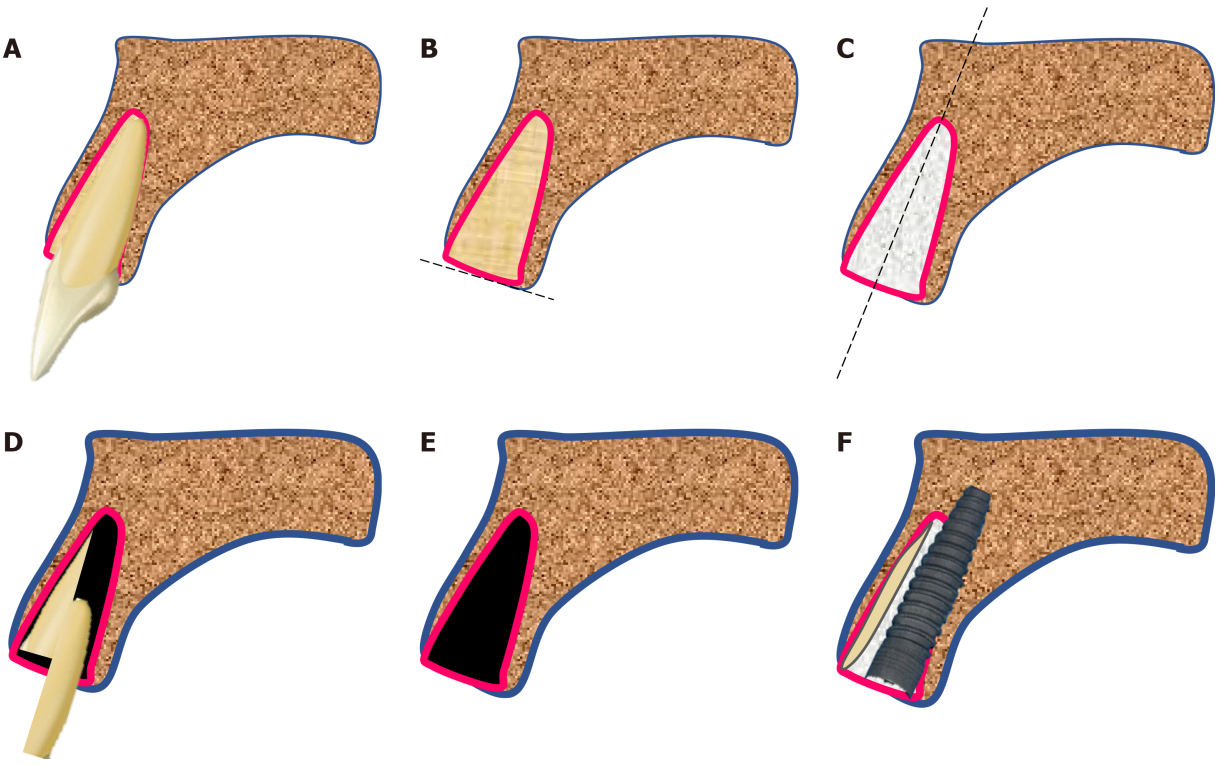


Figure 3 The 'Socket-shield' technique step-by-step. A: Tooth in bony socket; B: The tooth in concern is sectioned horizontally 1 mm above the bone crest; C: Root is sectioned longitudinally in facial and palatal halves; D: The palatal section is extracted; E: The facial root section is concaved with a long shank dental bur; F: Immediate placement of dental implant palatal to the retained root fragment. The jumping space if any can be grafted is possible.

that 8/12 roots had complete bone coverage on the cut surface, and the vitality of all the pulps was retained. In a histological study, Johnson *et al*[29] reported that the root fragments remained vital after 1 year. The study also found some roots achieving complete canal closure due to osteodentine formation.

Gray and Vernino[30] conducted an animal study in baboons to evaluate the effect of unintentional placement of the root form implants into or near retained root fragments. The study found that many implants were placed through the retained roots while others were placed through the PDL of the other root fragments. Histologically they did not find any inflammation at any site. At the locations where the implant was in direct contact with PDL, fibrous encapsulations of the implants were detected, while in some areas, calcified material was deposited on the implant surface.

Buser *et al*[31] and Hürzeler *et al*[14] evaluated the effects of placement of endosseous titanium implant fixture in the presence of retained roots in monkeys and Beagle dogs, respectively. Buser *et al*[31] reported that immediate implant placement over apical portions of the fractured retained roots resulted in cementum apposition between the fractured root and the implant surface. Hürzeler *et al*[14] modified this idea further and intentionally fractured the roots axially preserving the buccal portion of the root and placed an enamel matrix derivative in the socket before placing an implant. Similar results were reported showing that cementum covered the surface of both the retained root fragment and the implant. Bäumer *et al*[32] further evaluated the socket-shield using vertically separated root fragments in beagle dogs (without Emdogain). A new bone was found between the dentine layer and the implant surface; however, converse to the previous study, wherein Emdogain was coated on the root fragment, the present study did not find any cementum formation up to 4-mo follow-up period.

This might lead to the speculation that if one root fragment can prevent the resorption of buccal bone, then it would be beneficial to leave the root-membrane-type wall along all the socket walls and place the implant in the centre. Calvo-Guirado *et al*[33] conducted a histological animal study, wherein 36 implants were inserted in the mandible of 6 American Foxhound dogs using the principle of 'root-t-belt', *i.e.*, the crowns were sliced at the bone crest, and implant beds were prepared in the centre of the roots passing 3 mm apically. At the 3-mo histological evaluation, all fixtures were osseointegrated; however, three samples demonstrated inflammatory reaction, and some radicular fragments were detected in the resorption state.

Mitsias *et al*[34] presented histological evidence for a root-membrane case. The human sample analysis revealed that even after 5 years of post-implant placement, the buccal bone plate was perfectly maintained, and no resorption was evident. The buccal bone was supported and nourished by a healthy, intact PDL. Moreover, the implant showed good osseointegration, with a high amount of compact, mature bone on the surface. At the apical region of the root fragment, where the implant was in direct contact with the root membrane, the authors also noted cementum, which may have migrated from the root to the implant surface[34]. Schwarz *et al*[35] presented histological evidence of the integration of an implant in the dentin of an unintentionally retained root fragment. The authors discovered that subsequent to trauma during implant site preparation, a layer of reparative dentin was formed on the surface of the retained root fragment that bridged the space between the implant and the root fragment. This type of mineralized integration led them to coin the term 'dentointegration', and its pace was equivalent to that of osseointegration seen on the other parts of the implant[35].

Siormpas *et al*[20] presented one of the largest longitudinal studies on the survival of immediate implants placed adjacent to a root fragment. The data from 46 patients concluded that all implants successfully maintained osseointegration till the end of the follow-up period, giving a cumulative survival rate of 100%. Regarding the fate of the root fragment, the authors found only one patient with the apical root resorption, which was also self-arrested and did not interfere with the osseointegration of the dental implant. In addition, Bäumer *et al*[32] also raised a genuine clinical question as to what type of tissue would be formed following resorption of the retained root? A previous study demonstrated that the resultant space would be healed by bone fill. Bäumer *et al*[32] further conducted a study to evaluate whether the socket-shield technique could be successful if the buccal root fragment shows a vertical fracture line. Leaving the fracture line untreated is detrimental to the overall prognosis as it acts like a recess for bacteria, ultimately leading to infection. Therefore, the authors recommended surgical separation of the buccal shield into two halves along the fracture line. At the follow-up visit, the animal histological data showed a higher buccal alveolar crest height with healthy peri-implant soft tissue and no resorption at the apical end of the tooth fragment. The gap between the root fragment and the implant and the vertically drilled space between the two parts of the buccal shield was filled with the bone in the horizontal section[32].

Approximately, 4-wk are required post-extraction to cover the socket with epithelium. It may be assumed that a similar process occurs between the implant and the retained root fragment. Initially, a blood clot is filled between the implant and the buccal root fragment. This clot prevents the epithelium from growing along the internal root surface. Thus, it may seem that the cells from the remaining PDL are capable of colonizing the root surface and regenerating a new periodontal attachment.

COMPLICATIONS AND MANAGEMENT

In the previous root submergence technique, gingival tissue perforation and cyst formation were documented complications. To prevent perforation, the roots should be excised at a position slightly apical to the bony edge and beveled and smoothened to avoid any sharp edges.

The earliest histological finding showed a failed, plasma-sprayed titanium implant in contact with an undetected residual root presented with hypercementosis and no PDL[36]. The study also hypothesized that the unintentionally retained root might be a putative source of pathogenic bacteria from the PDL or the root canal itself, which compromises the osseointegration of the implant. Thus, it can be the potential cause of retrograde peri-implantitis, a term introduced by McAllister *et al*[37]. It is characterized by symptomatic periapical lesions with a healthy coronal bone-implant interface that develops within months of implant fixture placement.

In partial extraction therapy cases, where infection of the root fragment/section is coupled with mobility, removing the root membrane is mandatory. The mobility of the root shield, with or without infection, necessitates its removal. If the implant fails to osseointegrate, but the socket-shield is stable, immobile, and free of infection, the implant can be removed, leaving the shield in-situ for healing as a pontic-shield concept. The subsequent re-evaluation could be conducted to deduce whether an implant should be placed palatal to the shield or used as a pontic site.

FUTURE TRENDS AND ADVANCES

With additional literature that would be published on the success of socket-shield and pontic-shield techniques in the future, the procedure would be deemed an ideal requirement. Thus, there would be no more whole tooth extraction, and if not indicated, such whole tooth extraction would be deemed as an act of negligence.

Furthermore, simple and sophisticated technological advances would make the procedure easy and predictable. Thus, there is a dire need to develop a predictable and reproducible set of protocols for socket-shield and/or pontic-shield procedures.

CONCLUSION

The extraction of a tooth, which for long is regarded as a simple and uncomplicated procedure, should hereon be performed with an understanding that significant ridge resorption will follow. Surgical techniques performed later to compensate for the lost bone (guided bone regeneration/GTR/socket grafting/immediate implant) are not completely effective in preventing the alveolar bone resorption. Previous studies have shown that retaining the root fragment (along with its healthy PDL) is the most economical and successful therapy and should be recommended as required. However, the socket-shield/pontic-shield technique is a sensitive procedure, and its success depends on the operating clinician's expertise. The extraction of the whole tooth might be the choice of therapy when socket-shield or pontic-shield is not possible due to anatomical variations, infections, or lack of clinical expertise. Regardless of the size, whenever it is decided to leave a root fragment (or whole root for that matter) *in situ* (for whatever reasons), it is the dentist's ethical duty to advise/inform the patient and ensure repeated clinical, radiographic follow-up in the future.

Although socket-shield technique offers promising results, supporting clinical data are limited due to the lack of well-designed prospective randomised controlled studies. Thus, according to the review by Blaschke and Schwass[38], at this stage, it is not clear whether the socket-shield/pontic-shield techniques provide a long-term stable clinical outcome.

Further clinical research studies, preferably prospective randomised controlled trials involving power analysis to determine an adequate cohort size for statistical interpretation, would draw reliable conclusions.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 August 28; 9(4): 342-352

DOI: 10.13105/wjma.v9.i4.342

ISSN 2308-3840 (online)

SYSTEMATIC REVIEWS

Troponin I biomarker as a strong prognostic factor for predicting COVID-19 mortality: A systematic review

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Author contributions: Ashraf H, Soleimani A, Kazemi saeid A, Sadat Naseri A, Majidi F, Peirovi N, Karbalai Saleh S have contributed equally and provided critical feedback and helped shape the research, analysis, and manuscript.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

The increase in circulating Troponin-I in the blood of patients suffering coronavirus disease 2019 (COVID-19) can be a strong prognostic factor for predicting disease poorer outcome.

AIM

To review the literatures to approve this claim systematically.

METHODS

Two blinded reviewers independently screened the titles and abstracts of the manuscripts using the keywords and deeply searching the databanks including PubMed, Scopus, Google Scholar, and Web of knowledge, followed by profoundly appraisement of the full texts to assess the inclusion appropriateness.

RESULTS

The manuscripts entered into our final assessment were categorized as the two groups including 10 manuscripts describing and comparing death and disease-related complications between the subgroups of patients with raised serum troponin level and those with normal ranges of this biomarker and 7 manuscripts comparing the mean level of serum troponin concentration across the survived and non-survived groups. Comparing outcome of COVID-19 disease in the groups with raised troponin level and normal level of this markers showed increased the likelihood of death [hazard ratio (HR) = 4.967, $P < 0.001$], acute respiratory distress syndrome (HR = 5.914, $P < 0.001$), acute kidney injury (HR =

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Manuscript source: Unsolicited Manuscript

Specialty type: Cardiac and Cardiovascular Systems

Country/Territory of origin: Iran

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 28, 2021
Peer-review started: March 28, 2021
First decision: April 28, 2021
Revised: July 3, 2021
Accepted: August 24, 2021
Article in press: August 24, 2021
Published online: August 28, 2021

P-Reviewer: Azarbakhsh H, Peng D, Poggio PD, Wu HHL
S-Editor: Liu M
L-Editor: A
P-Editor: Li JH

3.849, $P < 0.001$), and intensive care unit (ICU) admission (HR = 3.780, $P < 0.001$) following raise of troponin. The pooled analysis showed significantly higher concentration of this marker in the survived group compared to non-survived group (weighted mean differences of 22.278, 95%CI: 15.647 to 28.927, $P < 0.001$).

CONCLUSION

Raising troponin-I on admission can be linked to the increase risk for in-hospital death, acute respiratory distress syndrome, kidney injury, and ICU admission by 4.9, 5.9, 3.8, and 3.7 times as compared to those with initial normal troponin-I concentration. Thus, raising baseline value of troponin-I can be used as a prognostic factor for poor outcome of COVID-19.

Key Words: Troponin-I; COVID-19; Mortality; Morbidity; Cardiac biomarker; Acute respiratory distress syndrome

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Core Tip: We systematically reviewed the literatures to assess this claim that an increase in troponin-I levels could be a prognostic factor in predicting disease severity and mortality in patients with coronavirus disease 2019. According to our findings, regardless of the history of myocardial injuries or the presence of cardiovascular risk profile, the value of troponin I should be accurately assessed on admission. Raising troponin-I on admission can be linked to the increase risk for in-hospital death, acute respiratory distress syndrome, kidney injury, and intensive care unit admission by 4.9, 5.9, 3.8, and 3.7 times as compared to those with initial normal troponin I concentration.

Citation: Ashraf H, Soleimani A, Kazemi saeid A, Sadat Naseri A, Majidi F, Peirovi N, Karbalai Saleh S. Troponin I biomarker as a strong prognostic factor for predicting COVID-19 mortality: A systematic review. *World J Meta-Anal* 2021; 9(4): 342-352
URL: https://www.wjgnet.com/2308-3840/full/v9/i4/342.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i4.342

INTRODUCTION

The new coronavirus known as coronavirus disease 2019 (COVID-19) has so far (15 February 15) infected more than 108 million people, leading to more than 2 million deaths. About 15 to 20 percent of patients experience a severe illness resulting in 2 to 3 percent mortality. The disease presents with acute respiratory syndrome with fever, dry cough, dyspnea and myalgia[1-4]. To date, there is no confirmed treatment for this disease. Therefore, accurate and early diagnosis and determination of its severity can prevent its further progression.

Along with acute respiratory failure as a prominent and debilitating manifestation of COVID-19, multidimensional organ defects have been also detected in the affected patients certainly resulting from the virus triggering role on pro-inflammatory cytokines activation and secretion, as well as coagulation abnormalities[5,6]. A complex of such events can predispose the patients to cardiovascular ischemic and thromboembolic events[7,8]. Some clinical data have supported the strong link of COVID-19 infection to cardiac and cerebrovascular ischemic events leading to high mortality and disabilities[9]. This infection is now suggested to promote myocardial injuries leading to cardiac arrhythmias, myocardial hypertrophy, acute coronary syndrome and even acute heart failure. We obvious a bidirectional interaction between the cardiovascular system and infection to COVID-19, however, the exact mechanisms responsible for such cardiovascular defects remains elusive. Some molecular-based studies emphasize the central role of pro-inflammatory cascades such as the activation of interleukin-6, interleukin-1beta, and tumor necrosis factor-α as the main flaring factors for such events[10]. Also, the presence of some specific receptors for the virus such as ACE2 as a gateway for the virus to enter tissues such as the myocardium seems necessary for the mentioned injuries[9]. There is also strong evidence on a close

association between the effects of the virus and underlying cardiovascular risk factors such as hypertension and diabetes so that the presence of any of these risk factors increases the risk of ischemic events many times over. The body of evidence highlights the high risk of ischemic heart disease such as myocardial infarction in patients with COVID-19[11,12].

Therefore, one of the relatively prevalent adversity in patients with COVID-19, particularly in the elderly and patients with prior predisposing factors like hypertension or diabetes mellitus is cardiovascular lesions in the form of ischemic heart attacks, arrhythmias, and vascular disorders. In this regard, considering that the increase in the level of cardiac biomarkers such as troponin-I as a marker of myocardial damage has been fully proven[13], it seems that in COVID-19 patients with myocardial and arrhythmic lesions, we see an increase in this marker. The increase in circulating troponin-I in the blood of patients may be also a prognostic factor for predicting the severity of the disease and its mortality[14]. In this study, we systematically reviewed the literature to assess this claim that an increase in troponin-I levels could be a prognostic factor in predicting disease severity and mortality in patients with COVID-19.

MATERIALS AND METHODS

For performing the present systematic review and meta-analysis, the full guideline of the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" was followed[15]. In the first step and after explaining the study's main question and specific goals, all prospective and retrospective comparative studies that evaluated the link between the serum level of troponin-I and two COVID-19 related parameters including disease severity and mortality were considered to be eligible for primary assessment. In this regard, deeply searching the manuscript databanks including PubMed, Scopus, Google Scholar, and Web of knowledge was planned from inception to October 2020; The main keywords were: 'covid-19' OR 'sars-cov-2' OR '2019-ncov' AND 'troponin' AND 'mortality' OR 'death'. In the searches, the review papers, case presentations, letter to editors, and abstracts without full text access were all excluded. Non-English studies were excluded from the meta-analysis. Also, in cases of lack of access to the full text of the articles, correspondence was made with the author in charge of the articles to obtain the full article, and in case of lack of access to the original article; it was removed from the study. The manuscript reviewing was done by two blinded reviewers, screening the titles and abstracts followed by profound appraisement of the full texts independently to assess the inclusion appropriateness. The presence of any disagreement between them was judged and checked again by another reviewer as the last arbiter. The eligibility and reasons for not including the papers are schematically presented in Figure 1. The bias hazard was blindly assessed by two authors using the Cochrane risk of bias tool, afore finalizing the meta-analysis. The level of bias was qualitatively classified into high, uncertain, or low bias[16]. Accordingly, the following domains are typically used to specify the level of bias: How to select the participants (selection bias), how to perform the measurement of troponin-I along the management of disease-related outcomes including disease severity and in-hospital death, and how to manage confounders and missing data. The permanent effects or random-effects (in case of significant heterogeneity across the data) models were used to obtained pooled relative risk (with 95% confidence interval and corresponding *P*-values) for disease severity and death due to troponin-I raising as well as to obtained pooled dichotomous data using the mean difference (MD) for the level of troponin-I. The incongruity between the studies was evaluated by determining I^2 values. A sensitivity analysis was also performed, in which observational studies at critical risk of bias were excluded from the analysis. Publication bias was also appraised by the rank correlation test and also affirmed by the funnel plot analysis. Reported values were two-tailed, and hypothesis testing results were assumed statistically significant at $P = 0.05$. We used the Comprehensive Meta-Analysis Software (CMA, version 3.0) for statistical analysis.

RESULTS

The study selection process is shown in Figure 1. On the field, 216 articles were prepared by the database searching at the beginning. We removed 137 articles as they were duplicated or unrelated to the subject of the systematic review. At first, 79 articles

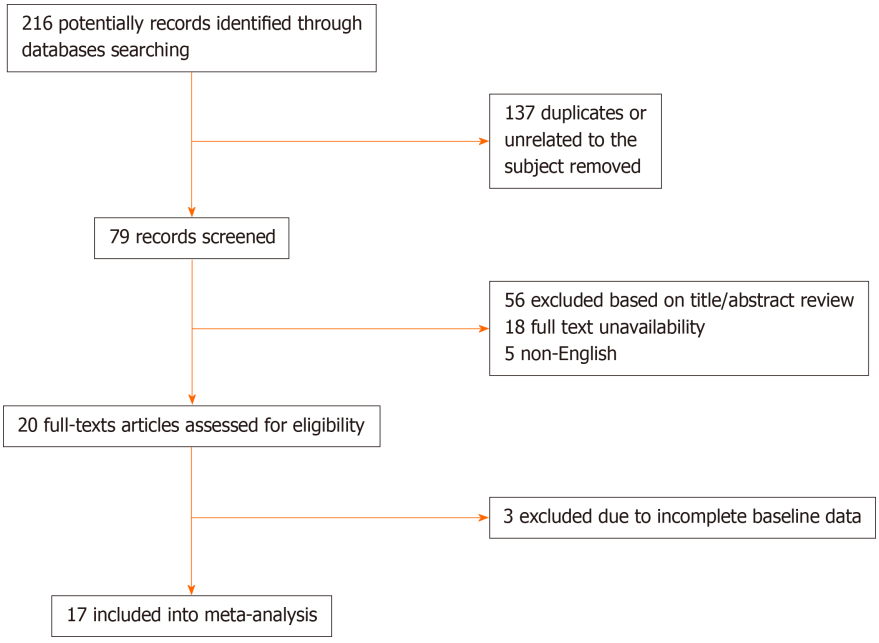


Figure 1 The flowchart of screening the eligible studies.

were initially under-screened. Fifty-nine articles were excluded based on the titles and abstracts. The extant 20 articles were specified for subsequent eligibility. We also excluded 3 more articles because the data and contents were not completed. Eventually, 17 articles were qualified for the final analysis[17-32]. Table 1 describes the baseline characteristics of the included studies. Evaluation of the publication and systematic bias demonstrated that approximately all articles were supposed as low risk or with unclear biases; hence, the obtained results could be considered valid and none of the articles was supposed to have a high risk of bias (Figure 2).

The manuscripts entered into our final assessment were categorized as the two groups including 10 manuscripts describing and comparing death and disease-related complications between the subgroups of patients with raised serum troponin level and those with normal ranges of this biomarker[17-26] and 7 manuscripts comparing the mean level of serum troponin concentration across the survived and non-survived groups[18-33]. The main point concerning the present meta-analysis was to first present the value of troponin with different laboratory units that of course could be matched through unit conversion. However, the different techniques employed for troponin value assessment, the type of study as prospective or retrospective, the different cutoff values considered for defining troponin abnormal raise as well as the time considering for patients' follow-up might lead to high heterogeneity across the two groups that were measured in our meta-analysis.

Overall, 10 studies assessed the early mortality and morbidity in the groups with and without raised serum troponin level (Table 1). In this regard, the outcome of COVID-19 was compared between the group with a normal troponin range (n = 4566) and the group with raised troponin level (n = 1846). The average age of participants in the two groups was 59.8 years and 71.2 years respectively. First, the overall rate of raising serum troponin level was estimated to be 32.2% (95%CI: 25.0% to 40.5%) in COVID-19 patients admitted to the hospitals. Comparing the outcome of COVID-19 disease in the groups with raised troponin level and normal level of this marker (Table 2) showed an increased likelihood of death [hazard ratio (HR) = 4.967, 95%CI: 2.883 to 8.557, P < 0.001], acute respiratory distress syndrome (ARDS) (HR = 5.914, 95%CI: 3.027 to 11.555, P < 0.001), acute kidney injury (HR = 3.849, 95%CI: 3.112 to 4.760, P < 0.001), and also intensive care unit (ICU) admission (HR = 3.780, 95%CI: 2.405 to 5.943, P < 0.001) following raise of troponin and thus the abnormal value of troponin on admission could effectively predict poor outcome in COVID-19 patients (Figure 3). The heterogeneity across the studies in the assessment of disease outcome was relevant with the I^2 values ranged 74.877 to 91.317.

In the second assessment concerning the difference in the value of troponin-I between survived and non-survived patients suffering COVID-19 (Table 3), The pooled analysis showed significantly higher concentration of troponin-I in the survived group compared to non-survived group (weighted MD of 22.278, 95%CI:

Table 1 The baseline details of studies included in our meta-analysis considering the groups with raised and normal values of troponin I

Ref.	Country	Population	Mean age	Male/female	Cutoff for TnI raising
Arcari *et al*[17]	Italy	Normal TnI: 39	66 ± 17	28/11	14 pg/mL
		Raised TnI: 64	79 ± 13	19/45	
Fan *et al*[18]	China	Normal TnI: 67	58 ± 15	38/29	0.01 μg/L
		Raised TnI: 22	71 ± 12	11/11	
Guo *et al*[19]	China	Normal TnI: 135	53 ± 13	57/78	99th percentile
		Raised TnI: 52	71 ± 9	34/18	
Lala *et al*[20]	United States	Normal TnI: 2206	66 ± 13	1312/894318/212	0.09 ng/mL
		Raised TnI: 530	68 ± 15		
Lombardi *et al*[21]	Italy	Normal TnI: 336	64 ± 13	234/102201/77	99th percentile
		Raised TnI: 278	71 ± 12		
Lorente-Ros *et al*[22]	Spain	Normal TnI: 560	63 ± 12	367/193	14 ng/L
		Raised TnI: 147	78 ± 14	77/70	
Raad *et al*[23]	United States	Normal TnI: 630	59 ± 11	280/350	18 ng/L
		Raised TnI: 390	70 ± 13	229/161	
Karbalai Saleh *et al*[24]	Iran	Normal TnI: 271	57 ± 15	167/104	99th percentile
		Raised TnI: 115	65 ± 15	69/46	
Shah *et al*[25]	United States	Raised TnI: 116	59 ± 14	70/123	0.05 ng/ml
		Normal TnI: 193	68 ± 14	62/54	
Singh *et al*[26]	United States	Normal TnI: 129	53 ± 11	54/75	17 ng/L
		Raised TnI: 132	71 ± 12	70/62	

15.647 to 28.927, $P < 0.001$). The statistical heterogeneity was significant with an I^2 of 99.123. We showed a significant publication bias as evidenced by either funnel plot asymmetry or the Egger test for all comparative analyses.

DISCUSSION

High-sensitive troponin-I has been well known as a strong and even specific cardiac biomarker for diagnosing ischemic cardiac injury as well as predicting its poorer outcome. The observations among critically ill patients who are suffering from COVID-19 especially those who require intensive care also suggested higher levels of this marker on admission as well as within hospitalization. In this regard, recent studies have focused this point that by initially measuring troponin-I in patients hospitalized due to definitive diagnosis of COVID-19, predicting outcomes of disease especially occurring in-hospital death and disease severity may be certainly possible. As shown in our systematic review, assessing the baseline level of troponin-I not only can strongly predict death in COVID-19 patients, but also it can be used as a valuable factor to predict disease sequels including ARDS, kidney injury, and requiring ICU admission. Due to this fact, the appearance of each of these complications are indicators for disease severity (particularly ICU admission). Therefore, raising in troponin-I is a valuable indicator for disease severity. Overall, as well indicated in our meta-analysis, raising troponin-I on admission can be linked to the increased risk for in-hospital death, ARDS, kidney injury, and ICU admission by 4.9, 5.9, 3.8, and 3.7 times as compared to those with initial normal troponin-I concentration. It should be noted that the use of this parameter along with other known predictive parameters can be more valuable to predict non-survived patients or those with disease-related adverse events. Despite demonstrating the value of troponin-I as a strong predictor for COVID-19 poorer outcomes, our results exposed to a high heterogeneity across the findings. Such heterogeneity can be explained by first the difference insignificant

Table 2 The outcome of coronavirus disease 2019 considering the groups with raised and normal values of troponin

Ref.	Raised TnI rate	Death rate	ARDS	Kidney injury	ICU admission
Arcari *et al*[17]	39/103	Raised TnI: 12	---	---	---
		Normal TnI: 7			
Fan *et al*[18]	22/89	---	Raised TnI: 20	Raised TnI: 9	---
			Normal TnI: 12	Normal TnI: 10	
Guo *et al*[19]	52/187	Raised TnI:3 1	Raised TnI: 30	Raised TnI: 14	Raised TnI: 31
		Normal TnI: 12	Normal TnI: 16	Normal TnI: 4	Normal TnI: 14
Lala *et al*[20]	530/2736	Raised TnI: 223	---	---	---
		Normal TnI: 102			
Lombardi *et al*[21]	278/614	Raised TnI: 102	---	Raised TnI: 41	---
		Normal TnI: 43		Normal TnI: 13	
Lorente-Ros *et al*[22]	147/707	Raised TnI: 60; Normal TnI: 130	---	---	Raised TnI: 7Normal TnI: 5
Raad *et al*[23]	390/1020	Raised TnI: 128	Raised TnI: 93	Raised TnI: 224	Raised TnI: 105
		Normal TnI: 52	Normal TnI: 64	Normal TnI:178	Normal TnI:93
Karbalai Saleh *et al*[24]	115/386	Raised TnI: 47	Raised TnI: 46	Raised TnI: 35	Raised TnI: 41
		Normal TnI: 30	Normal TnI: 30	Normal TnI: 25	Normal TnI: 38
Shah *et al*[25]	116/309	Raised TnI: 44	---	Raised TnI: 13	Raised TnI: 58
		Normal TnI: 22		Normal TnI: 5	Normal TnI: 52
Singh *et al*[26]	132/276	Raised TnI: 20	Raised TnI: 29	---	Raised TnI: 63
		Normal TnI: 5	Normal TnI: 10		Normal TnI: 25

Table 3 The baseline details of studies included in our meta-analysis considering the survived and non-survived groups

Ref.	Country	Population	Mean age	Male/female	Mean TnI
Aladağ and Atabey[27]	Turkey	Survived: 35	68 ± 14	22/13	Survived: 0.001 ng/mL
		Non-survived: 15	68 ± 15	6/9	Non-survived: 0.010 ng/mL
Deng *et al*[28]	China	Survived: 212	62 ± 11	97/115	Survived: 0.006 ng/mL
		Non-survived: 52	74 ± 15	33/19	Non-survived: 0.051 ng/mL
Liberati *et al*[15]	Italy	Survived: 425	61 ± 13	287/138	Survived: 0.006 ng/mL
		Non-survived: 98	76 ± 10	68/30	Non-survived: 0.036 ng/mL
Nie *et al*[29]	China	Survived: 200	60 ± 11	180/20	Survived: 0.002 ng/mL
		Non-survived: 111	72 ± 12	88/23	Non-survived: 0.032 ng/mL
Rath *et al*[30]	Germany	Survived: 107	67 ± 15	65/42	Survived: 0.014 ng/mL
		Non-survived: 16	73 ± 16	12/4	Non-survived: 0.024 ng/mL
Shi *et al*[31]	China	Survived: 609	61 ± 13	287/322	Survived: 0.006 ng/mL
		Non-survived: 62	74 ± 15	35/27	Non-survived: 0.023 ng/mL
Yang *et al*[32]	China	Survived: 145	57 ± 13	77/68	Survived: 0.004 ng/mL
		Non-survived: 58	67 ± 15	38/20	Non-survived: 0.020 ng/mL

divergent in the cutoff points defined for troponin-I raising, also by the difference in the baseline characteristics of study populations especially with respect to the presence of cardiovascular risk profiles, the sample size of the studies, the time for patients' following–up, as well as the techniques for measuring troponin-I concentration. However, despite such heterogeneities across the studies, this marker should also be considered as an important and strong predictor of disease consequences. In a meta-

Ref.	Patient selection	Index test	Measurement	Managing confounders
Arcari *et al*[17]	+	?	?	+
Fan *et al*[18]	?	?	?	+
Guo *et al*[19]	?	+	+	+
Lala *et al*[20]	+	+	+	+
Lombardi *et al*[21]	?	+	+	+
Lorente *et al*[22]	+	+	+	+
Raad *et al*[23]	+	?	?	+
Saleh *et al*[24]	+	+	+	+
Shah *et al*[25]	+	+	+	+
Singh *et al*[26]	?	?	+	+
Aladağ and Atabey[27]	+	+	+	+
Deng *et al*[28]	+	?	+	+
Michela *et al*[15]	+	+	+	+
Nie *et al*[29]	+	+	?	?
Rath *et al*[30]	+	+	?	?
Shi *et al*[31]	+	+	+	+
Yang *et al*[32]	?	?	?	+

+	?	-
Low	Unclear	High

Figure 2 The Assessment of the risk of bias.

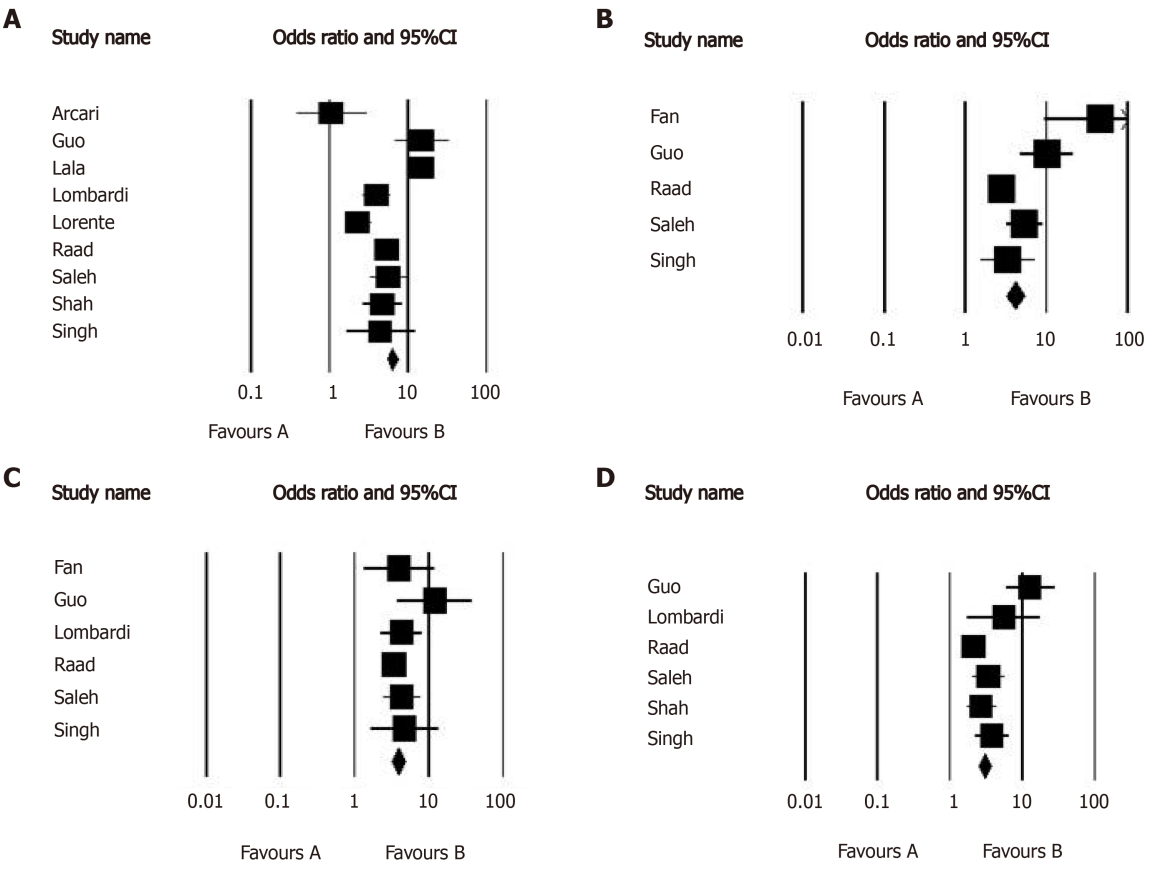


Figure 3 The hazard ratio in coronavirus disease 2019 related death. A: Acute respiratory distress syndrome; B: Acute kidney injury; C: ICU admission; D: In the group with raised troponin as compared to group with normal level of this marker in pooled assessment of the studies.

analysis by Lippi *et al*[33] demonstrated a significant association between COVID-19 severity and elevated troponin-I level, despite their high heterogeneity.

Regarding the association between the raise of troponin-I and COVID-19 outcome, some probable mechanisms have been delivered. Firstly, it has been revealed that the patients with raised troponin-I level had higher rates of cardiovascular risk factors that

are now shown to be closely linked to the increased risk for death and severity of COVID-19[19]. Also, any severe respiratory viral infections especially when lead to sepsis are also associated with increasing the level of troponin-I. Recently, a quadrilateral mediator loop is revealed describing the association between raising troponin levels and disease outcome[34,35]. These four loops include: (1) Secretion of pro-inflammatory cytokines that raised favored by ACE2 receptor suppression and induced oxidative stress and endothelial dysfunction; (2) Microangiopathy and prothrombotic states usually stimulated by both oxidative stress and endothelial dysfunction; (3) Myocardial infarction induced directly by viral invasion or by inflammatory cascades activation; and (4) Myocarditis by inflammatory reactions[36].

CONCLUSION

In conclusion, according to our findings, regardless of the history of myocardial injuries or the presence of cardiovascular risk profile, the value of troponin-I should be accurately assessed on admission because of its high predicting value for COVID-19 related mortality and morbidity.

ARTICLE HIGHLIGHTS

Research background

Troponin-I on admission has a high predicting value for coronavirus disease 2019 (COVID-19) related mortality. Troponin-I on admission has a high predicting value for COVID-19 related morbidity. Troponin-I can strongly predict disease sequels including acute respiratory distress syndrome (ARDS), kidney injury, and Intensive care units (ICU) admission requirement.

Research motivation

Accurate and early diagnosis and determination of COVID-19 severity can prevent its further progression. The increase in circulating troponin-I in the blood of patients suffering COVID-19 can be a strong prognostic factor for predicting disease poorer outcome. We systematically reviewed the literatures to approve this claim.

Research objectives

The increase in circulating troponin-I in the blood of patients suffering COVID-19 can be a strong prognostic factor for predicting disease poorer outcome.

Research methods

Deeply searching the manuscript databanks was planned. All studies that evaluated the link between the serum level of troponin-I and two COVID-19 related parameters including disease severity and mortality were considered to be eligible for primary assessment. The review papers, case presentations, letter to editors, non-English studies, and abstracts without full text access were all excluded. The manuscript reviewing was done by two blinded reviewers, screening the titles and abstracts followed by profound appraisement of the full texts independently to assess the inclusion appropriateness. The presence of any disagreement between them was judged and checked again by another reviewer as the last arbiter.

Research results

Comparing outcome of COVID-19 disease in the groups with raised troponin level and normal level of this markers showed increased the likelihood of death [hazard ratio (HR) = 4.967, $P < 0.001$], acute respiratory distress syndrome (HR = 5.914, $P < 0.001$), acute kidney injury (HR = 3.849, $P < 0.001$), and ICU admission (HR = 3.780, $P < 0.001$) following raise of troponin. The pooled analysis showed significantly higher concentration of this marker in the survived group compared to non-survived group (weighted mean differences of 22.278, 95%CI: 15.647 to 28.927, $P < 0.001$).

Research conclusions

In conclusion, according to our findings, regardless of the history of myocardial injuries or the presence of cardiovascular risk profile, the value of troponin-I should be accurately assessed on admission because of its high predicting value for COVID-19

related mortality and morbidity.

Research perspectives

The value of troponin-I should be accurately assessed on admission because of its high predicting value for COVID-19 related mortality and morbidity.

ACKNOWLEDGEMENTS

We are indebted to Research Development Center of Sina Hospital for their technical help.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 August 28; 9(4): 353-376

DOI: 10.13105/wjma.v9.i4.353

ISSN 2308-3840 (online)

META-ANALYSIS

Systematic review with meta-analysis of the epidemiological evidence in Europe, Israel, America and Australasia on smoking and COVID-19

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Author contributions: Lee PN conceived the study, checked the data entry and analysis, and wrote the paper; Coombs KJ carried out the literature searches and assisted in finalizing the paper; Hamling JS checked the literature searches, carried out the data entry and analysis, and assisted in finalizing the paper.

Supported by British-American Tobacco (Investments) Ltd., No. 5700014230; Japan Tobacco International S.A., No. 4700562109; and Philip Morris Products S.A., No 5700131814.

Conflict-of-interest statement: The authors have carried out consultancy work for many tobacco organizations.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2020 statement, and the manuscript was prepared and revised according to the PRISMA 2020 statement.

Open-Access: This article is an open-access article that was

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Abstract

BACKGROUND

Previous meta-analyses related smoking to death or severe infection from coronavirus disease 2019 (COVID-19) in hospitalized patients, but considered only a few studies, did not adjust for demographics and comorbidities, and inadequately defined smoking.

AIM

To review and meta-analyse epidemiological evidence on smoking and COVID-19, considering a range of endpoints, populations and smoking definitions and the effect of adjustment.

METHODS

Studies were identified from publications in English up to 30 September, 2020 involving at least 100 individuals, carried out in Europe, Israel, America or Australasia, not restricted to those with specific other diseases, and providing information relating smoking to various COVID-related endpoints. Meta-analyses were carried out for combinations of population and endpoint, with variation studied by smoking definition, adjustment level and other factors.

RESULTS

From 96 publications, 74 studies were identified, 37 in the United States, 10 in the United Kingdom, with up to four in the other countries. Three involved over a million individuals, and 37 involved less than a thousand. Adjusted results for smoking were available in 42 studies, with adjustment not considered in 20 studies. Results were considered by endpoint. No significant effect of smoking on COVID-19 positivity was seen in the general population, but there was a reduced

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Manuscript source: Unsolicited manuscript

Specialty type: Infectious diseases

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): E

Received: April 3, 2021
Peer-review started: April 3, 2021
First decision: June 18, 2021
Revised: June 28, 2021
Accepted: August 23, 2021
Article in press: August 23, 2021
Published online: August 28, 2021

P-Reviewer: Samadder S
S-Editor: Liu M
L-Editor: Webster JR
P-Editor: Ma YJ

risk in those tested. Best-adjusted estimates for current (*vs* never) smoking were 0.87 (95% confidence interval: 0.52-1.47) in the general population and 0.52 (0.43-0.64) in those tested. For those hospitalized due to COVID-19, unadjusted rates were significantly increased in current smokers (1.20, 1.01-1.42) and ever smokers (1.64, 1.41-1.91), but those adjusted for comorbidities showed no increase for current (0.82, 0.52-1.30) or ever smokers (1.00, 0.76-1.32). There was little evidence to suggest that smoking was associated with intensive care admission. For those hospitalized with COVID-19, best-adjusted estimates were 0.88 (0.72-1.08) for current smokers and 1.10 (0.99-1.22) for ever smokers. In those hospitalized with COVID-19, smoking was not significantly related to subsequent mechanical ventilation, with best-adjusted estimates of 1.12 (0.60-2.09) for current smokers and 1.05 (0.88-1.25) for ever smokers. For those hospitalized with severe COVID-19, best-adjusted estimates were 0.74 (0.49-1.12) for current smokers and 1.15 (0.87-1.51) for ever smokers; few estimates were adjusted for comorbidities. While smoking was associated with increased mortality in unadjusted analyses, the association disappeared after adjustment for comorbidities. For example, in those hospitalized with COVID-19, the unadjusted estimate for ever smokers of 1.59 (1.37-1.83) reduced to 1.07 (0.82-1.38) when adjusted for comorbidities. Studies on those with severe COVID-19 showed that smoking tended to be associated with worsening of the disease. However, no estimate was adjusted, even for demographics. Estimates did not clearly vary by location or study size, and there was too little evidence to usefully study variations by age, amount smoked or years quit.

CONCLUSION

The increased COVID-19 death rate in smokers seen in unadjusted analyses disappears following adjustment for demographics and comorbidities. Among those tested, smoking is associated with lower COVID-19 infection rates.

Key Words: Smoking; COVID-19; Meta-analyses; Review; Europe; America

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Core Tip: Detailed analyses of 74 studies related smoking to being tested for coronavirus disease 2019 (COVID-19), having COVID-19, or suffering death or severe disease due to COVID-19. Various smoking indices were studied, as were the effects of adjusting for other factors. Although many studies provided limited unreliable results, consistent evidence showed that of those tested, smokers were less likely to have COVID-19. Among those positive for or hospitalized with COVID-19, there was a clear association between smoking and COVID-19 death and severity in unadjusted analyses, which disappeared following adjustment for comorbidities and demographics. Any adverse effects in smokers appear to derive from their poorer prior health status.

Citation: Lee PN, Hamling JS, Coombs KJ. Systematic review with meta-analysis of the epidemiological evidence in Europe, Israel, America and Australasia on smoking and COVID-19. *World J Meta-Anal* 2021; 9(4): 353-376
URL: https://www.wjgnet.com/2308-3840/full/v9/i4/353.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i4.353

INTRODUCTION

In a previous project commenting on publications on smoking and coronavirus disease 2019 (COVID-19), we considered over 100 papers published up to the end of September 2020. Among these were various meta-analyses falling into two groups.

Eight publications[1-8] considered smoking prevalence in hospitalized patients, generally agreeing it was substantially less than expected from national statistics. This evidence does not necessarily show smoking protects against acquiring COVID-19.

Smoking may be markedly under-reported in studies based on medical records. Also, among those with COVID-19, smokers might be less likely than non-smokers to be hospitalized. These meta-analyses ignored relevant information from studies of those tested for COVID-19, or of the general population, where smoking habits were collected pre-pandemic, as well as more recent studies of hospitalized patients.

The other meta-analyses[3,6,9-28] concerned hospitalized patients, relating smoking to severity, progression or death from COVID-19, mainly from studies in China. While these generally reported positive associations which were often statistically significant, many meta-analysis estimates were unadjusted even for age, with comorbidities present pre-pandemic rarely considered. These meta-analyses also varied on the index of smoking used, which was not always clearly defined.

Here we describe meta-analyses aimed at avoiding the limitations of the early meta-analyses by considering more studies, not limiting attention to hospitalized patients, and paying particular attention to the definition of smoking and the effects of adjustment, as well as the reliability of the smoking data.

To limit the scope of the study and provide timely results various restrictions to the studies were made, as described in the methods section. Notably studies in China and other parts of Asia, except for Israel, were excluded. A recent review[29] classified few studies from Asia as "good" or "fair", most having much missing data and/or not reliably distinguishing current, former, ever and never smoking status. We were also aware of a large study in Israel[30] without these weaknesses.

Due to the more comprehensive data included, and better quality of the studies considered, the meta-analyses we describe should provide much better insight into the relationships of smoking to various COVID-related outcomes than do the meta-analyses referred to above.

MATERIALS AND METHODS

Full details of the methods used are given in Supplementary material 1 and are summarized below.

Study inclusion and exclusion criteria

Pre-defined criteria stipulated for practical reasons that studies should be described in English and were detected in searches up to September 2020. They should also be conducted in Europe, Israel, America, or Australasia, as discussed above. America here includes all the countries in South and Central America, as well as the United States and Canada. Studies restricted to individuals with specific other diseases were excluded as being less generalizable. Studies of less than 100 individuals were excluded as they provided inadequate power to detect reliable results, and as there were adequate numbers of larger studies. Studies should provide information relating smoking to the probability of one or more of the following relevant endpoints: being tested for COVID-19, having confirmed COVID-19, having self-reported COVID-19, being hospitalized with COVID-19, requiring mechanical ventilation for COVID-19, requiring intensive care for COVID-19, having severe/progressive COVID-19, or dying from COVID-19 or from any cause. The studies may concern various at-risk populations, including the general population, those tested for COVID-19, those positive for COVID-19, those hospitalized with COVID-19, or those with severe COVID-19.

Literature searches

As part of our earlier project, we carried out a first PubMed search on April 7, 2020, and then carried out further daily searches up to September 30, 2020.

Publications identified in our study as being of initial interest were then examined to identify studies satisfying our inclusion/exclusion criteria, and relevant meta-analyses. The meta-analyses were then examined for additional relevant studies meeting our inclusion criteria. Further studies were then sought from meta-analyses identified in further searches, from a further more detailed look at our original searches, and from examining reference lists of publications identified as relevant.

Multiple publications from the same study

To avoid double-counting results from the same study reported in multiple publications, the relevant publications were examined to identify publications from the same study.

Data recorded

Data from each publication were entered onto a study database and a linked effect estimate database. The study database recorded information on: publications considered; study title; study location; sexes, ages and races considered; study dates; study type; nature of population studied; sample size; definition of severe COVID-19 (if relevant); method of COVID-19 diagnosis; whether adjusted effect estimates were available; the confounding variables studied; and whether adjusted results on smoking were reported and, if not, why not (*e.g.*, smoking not significant in the adjusted model). It also recorded information on the smoking index for which results were available (*e.g.*, current *vs* never smoking), the source of the smoking data (*e.g.*, medical records), the extent of missing data, the percentage of smokers in the population studied, and whether dose-response data were available, as well as details of the endpoints and at-risk populations studied.

The effect estimate database included details of each individual effect estimate entered. Effect estimates were entered for every available combination of endpoints within a population, smoking index and level of adjustment (separated into unadjusted (U), adjusted for demographics only (D), adjusted also for comorbidities but not post-infection variables (C), and adjusted also for variables including post-infection responses to COVID-19 (P). Where available, effect estimates were entered by sex, age group and smoking dose (amount, duration, time since quitting). Other factors recorded included the publication the effect estimate was derived from; the population and endpoint considered; the smoking comparison; the type of effect estimate [odds ratio (OR), relative risk (RR) or hazard ratio (HR)]; the adjustment factors considered; the number of cases and at-risk subdivided by the smoking variable; the effect estimate and its lower and upper 95% confidence interval; and whether the estimate was given in the paper or was derived from the data presented. Derivation could be from the 2 × 2 table of numbers for unadjusted data, or using the method described by Hamling *et al*[31] to derive adjusted estimates for other smoking indices from estimates given in the publication (*e.g.*, ever *vs* never estimates from those for current *vs* never and former *vs* never).

All data were entered by Hamling JS or Coombs KJ and checked by Lee PN, with any disagreements discussed and resolved.

Meta-analyses

For each studied combination of endpoint within a population (*e.g.*, died while hospitalized with COVID-19), meta-analyses were carried out relating the endpoint to each of six indices of smoking; ever *vs* never, current *vs* non-current, current *vs* never, former *vs* never, a combined index most closely approximating to current *vs* never, and a combined index most closely approximating to ever *vs* never. The combined index for current *vs* never smoking includes, from each study reporting the endpoint/population combination, results in the following preference order (most to least preferred) – current *vs* never, current *vs* non-current, smoker (undefined) *vs* non-smoker, tobacco use *vs* none, ever *vs* never, and former *vs* never. The combined index for ever *vs* never smoking uses the preference - ever *vs* never, former *vs* never, smoker (undefined) *vs* non-smoker, tobacco use *vs* none, current *vs* never, and current *vs* non-current.

For each endpoint within a combination of population and smoking index, the results to be meta-analysed were selected using a first preference on level of adjustment and then a second preference on type of effect estimate. For adjustment, the order of preference (first to last) was adjustment for factors including comorbidities, adjustment for demographics only, unadjusted, and adjustment for factors measuring responses to COVID-19 infection (the lowest preference, as this may be a form of over-adjustment). For type of effect estimate, the preference order was HR, then RR, then OR.

Where the numbers of estimates permitted, the meta-analyses compare estimates by level of adjustment, type of estimate, sex, location and study size.

Statistical analysis

Fixed-effect and random-effects meta-analyses were conducted using the method of Fleiss and Gross[32] with heterogeneity quantified by H, the ratio of the heterogeneity chisquared to its degrees of freedom. H is directly related to the I^2 statistic[33] by the formula $I^2 = 100(H-1)/H$. For all meta-analyses, Egger's test of publication bias[34] was included.

All analyses were carried out using RoeLee release 63, build 52, available from RoeLee Statistics Ltd (www.roelee.co.uk).

RESULTS

Literature searches

Figure 1 summarizes the results of the literature searches, with fuller details, including reasons for rejecting papers (Supplementary material 2). Overall, 98 publications met the selection criteria.

Studies and study characteristics

Twelve references represented multiple publications from the same study. Allowing for this, data were entered for 76 separate studies. Subsequently, during data entry, it became apparent that two studies[35,36] provided no data for any of the endpoints considered, and only compared smoking prevalence with published data in the population at large. These studies were not further evaluated.

Supplementary material 3 summarizes the details for the 74 studies, including references. Studies were identified by the six character codes shown there.

Thirty-seven studies were from the United States, 10 from the United Kingdom (including seven restricted to England), four each from France, Israel, Italy, Mexico and Spain, two from Switzerland and one each from Australia, Brazil and Denmark. Also one study was conducted in the United Kingdom and Italy, and one in multiple European countries.

In 31 studies, the population considered was patients hospitalized with COVID-19, with a further four patients admitted to the intensive care unit (ICU). Also 19 studies considered those with a positive COVID-19 test, 11 included those who were tested for COVID-19 and seven included the general population. One study included hospital patients and non-COVID-19 controls, while another included those tested for COVID-19 as well as control groups not tested.

All studies included both sexes, and none selected individuals on race or ethnicity. One study was restricted to adults aged 47-87 years, with a further 41 restricted to adults, generally with a minimum age of 18 years, but sometimes having lower limits ranging from 15 years to 23 years. The remaining 32 studies did not refer to any age restriction. As shown in Supplementary material 3, the number of individuals with smoking data varied widely between the studies. The largest was OPENSA in England involving over 16 million individuals, with two other United Kingdom studies over a million, and three others over 100000. In contrast, 37 studies involved less than 1000 individuals.

It is also shown in Supplementary material 3 that some studies reported analyses for subsets of their populations, and for various endpoints. These endpoints included being tested for COVID-19, having confirmed or having self-reported COVID-19, being hospitalized with COVID-19, requiring intensive care or mechanical ventilation, having severe or progressive COVID-19, and mortality, either from COVID-19 or from all causes combined.

Thirty-six studies allowed calculation of separate effect estimates for current, former and ever smokers. Eight reported results for current smoking only, 19 for smoking history only, and eight for smoking undefined. One (TWIGG) reported results for tobacco use undefined, one (CHAND) had two source papers - one providing results for current smokers only, one for ever smokers only - and another (GUPTA) gave numbers of current or former smokers combined and adjusted results for current *vs* non-current smokers. The smoking data were mainly extracted from medical records, although in some cases the data came from a questionnaire or other sources, with no details given in six studies. Percentages with missing data on smoking were available in 35 studies, with 13 being over 20%.

Adjusted results for smoking were presented for 42 studies. In 12, only predictors other than smoking appeared in the adjusted model, either because smoking was not significant in univariate analyses, so was not considered in multivariate analyses, or because smoking dropped out of the multivariate modelling. In 20 studies, no adjusted results were presented.

In 54 studies, COVID-19 diagnosis was by reverse transcriptase-polymerase chain reaction or simply by polymerase chain reaction, but there were various alternatives, as indicated in Supplementary material 3.

Effect estimates available

Effect estimates were available for all but one study (KNIGHT), which stated only that "Ever cigarette smoking was predictive of death ($P < 0.05$)" without providing quantitative detail.

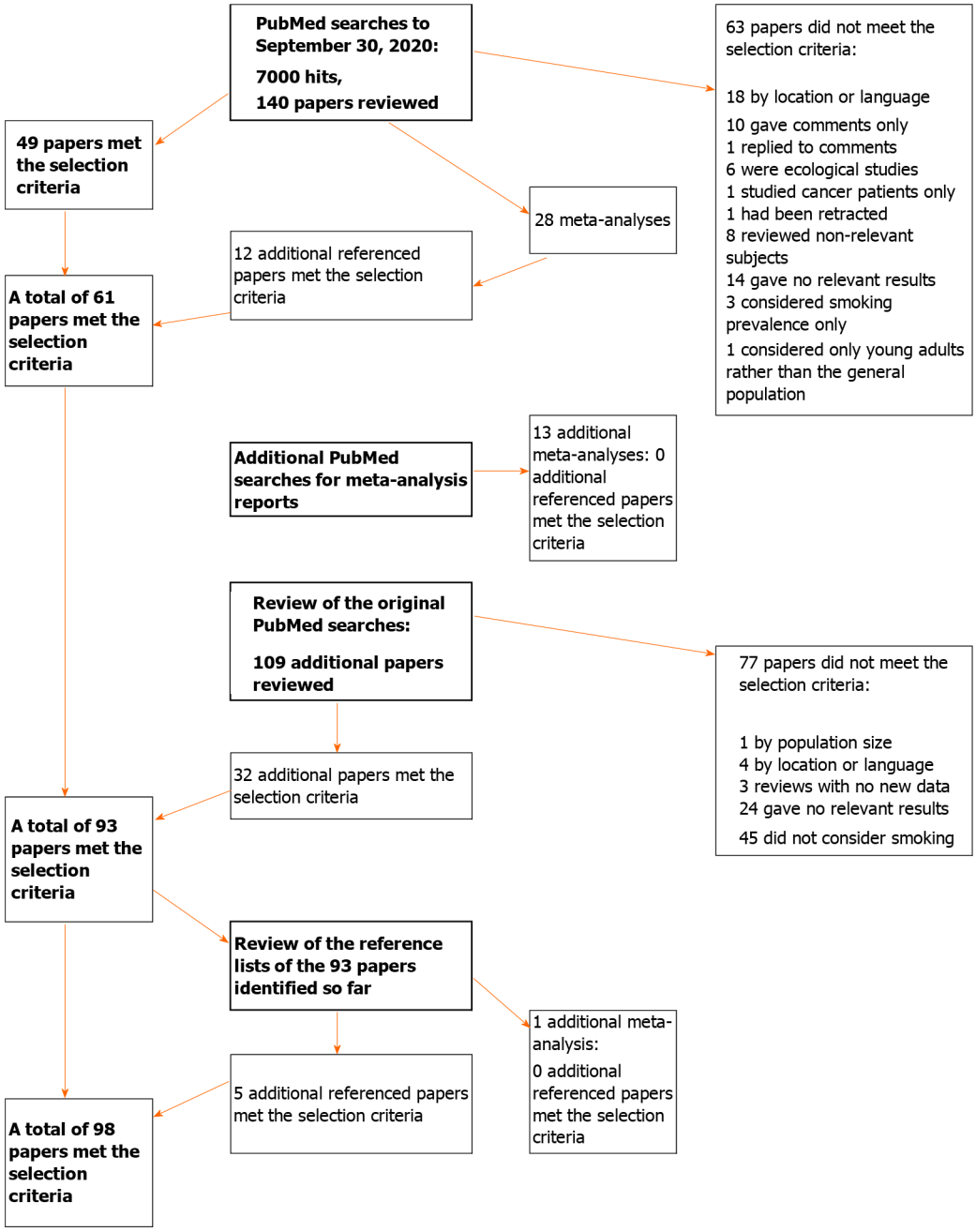


Figure 1 Flowchart of literature search. The flowchart shows, for each of the four stages of the process, the numbers of papers and meta-analyses considered, together with the numbers of papers meeting the selection criteria or rejected, with reasons for rejection shown.

Overall there were 738 effect estimates: 548 ORs, 122 RRs and 68 HRs. The studies providing most estimates were GU (100), BIOBNK (86) and VETERA (52), with 18 other studies providing 10 or more estimates. Fourteen studies provided only one estimate. There were 153 estimates for current *vs* never smoking, 202 for current *vs* non-smoking, 142 for former *vs* never smoking, 223 for ever *vs* never smoking, 16 for smoker *vs* non-smoker not otherwise stated, and two for tobacco *vs* no-tobacco not otherwise stated. One study (HIPPIS) provided 15 effect estimates by amount smoked, and one (TOOLKI) provided four estimates by years quit. No other study provided dose-response data.

Of the 738 estimates, 432 (58.5%) were unadjusted, 110 (14.9%) adjusted for demographics only, 169 (22.9%) adjusted for variables including co-morbidities but not responses to COVID-19, and 27 (3.7%) adjusted for a list including responses to COVID-19.

One study (YANOVE) provided effect estimates subdivided jointly by sex and age group. Four others (BIOBNK, GDEM, HOPKIN, MIYARA) provided estimates subdivided by sex only, and three others (GDEM, SINAI, VETERA) provided

estimates subdivided by age group only.

The at-risk population was the general population for 155 (21.0%) effect estimates, those tested for COVID-19 for 106 (14.4%), those positive for COVID-19 for 222 (30.1%), those hospitalized with COVID-19 for 188 (25.5%), and those in intensive care for 22 (3.0%). Other populations were considered for 45 (6.1%) effect estimates, with GU providing 36 of these, all based on populations representing a combination of a group considered above (*e.g.*, tested for COVID-19) and unmatched controls. Other such populations included a mixture of those positive for COVID-19 and those untested (two estimates from BIOBNK), those hospitalized but including non-COVID cases (five from MEINI), those tested for COVID-19 but not hospitalized (one from ADORNI) and those hospitalized with COVID-19 but not in ICU (one from PELLAU).

The endpoints considered in the 738 effect estimates were tested for COVID-19 in 41 (5.6%), positive for COVID-19 in 189 (25.6%), hospitalized for COVID-19 in 128 (17.3%), in intensive care in 92 (12.5%), mechanical ventilation in 58 (7.9%), severe COVID-19 (defined variously) in 58 (7.9%), and died in 172 (23.3%).

The effect estimates concerned many different combinations of endpoint within population, the commonest being hospitalized among those positive for COVID-19 (98 effect estimates), positive within those tested (90), died among those hospitalized (82) and positive among the general population (79).

The results below are considered by endpoint and within endpoint by population. Meta-analysis results are shown in the main tables for endpoint/population combinations that have data from at least five studies, with the individual study data summarized in Supplementary material 4 for combinations with data from fewer than five studies. Fuller details, including the individual effect estimates meta-analysed, the extent of heterogeneity between the estimates, and results of tests for publication bias, are provided in Supplementary material 5.

Endpoint: Tested for COVID-19

Four studies provided effect estimates (Supplementary Table 1). GU compared tested individuals with unmatched controls, and the remaining studies (BIOBNK, ADORNI and HOPKIN) compared those tested and not tested. BIOBNK provided estimates from multiple publications, the results from two being shown in Supplementary Table 1, with the others providing little extra information.

The results presented are somewhat conflicting, with the BIOBNK study reporting estimates above 1.00, generally significant, and tending to decrease with increasing adjustment, while ADORNI and HOPKIN, both only provided unadjusted results, with significant estimates below 1.00. GU shows a reduced probability of testing for current smoking and an increased probability of testing for former and ever smoking, with the estimates reducing with increasing adjustment.

Meta-analysis of this conflicting data was not attempted.

Endpoint: Positive for COVID-19

Table 1 presents the meta-analysis results from six studies where the at-risk population was the general population, and 15 where it was those tested for COVID-19. The "best-adjusted" results are those where, for each study, an effect estimate was selected in the order of preference C, D, U and P for level of adjustment. Results are shown for all the best-adjusted results and for those where C and U were the best-adjusted results available, which form the great majority of the best-adjusted results. The "all estimates U" results give estimates for the totality of unadjusted results, including those not included in the best-adjusted results being superseded by results for the same study with level of adjustment C or D. The results are consistent with no effect of smoking on positivity in the general population, but a reduced risk of positivity, particularly among current smokers, in those tested for COVID-19. No clear effects of adjustment were seen in either analysis.

Four other studies presented results for COVID-19 positivity based on other populations (Supplementary Table 2). In BIOBNK, COVID-19 positives were compared with the untested population, in ADORNI the population was those non-hospitalized, in MEINI the population was those hospitalized, whether or not from COVID-19, while GU compared tested individuals and unmatched controls. Although the results from BIOBNK suggested smokers were more likely to be positive, those from the other studies did not, the results for current smokers showing a negative association.

Endpoint: Hospitalized for COVID-19

Table 2 shows the meta-analysis results based on 19 studies of those positive for

Table 1 Main results for endpoint: Tested positive for coronavirus disease 2019

Selection of estimates[1]		Statistic[2]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to never
Population = general[3]								
Best-adjusted	All	EE (95%CI)	1.04 (0.79-1.37)	0.76 (0.56-1.02)	0.87 (0.52-1.47)	1.09 (0.94-1.28)	0.78 (0.57-1.06)	0.89 (0.73-1.09)
		n	5	7	5	5	7	7
	C	EE (95%CI)	1.10 (0.70-1.72)	0.93 (0.43-2.01)	0.96 (0.42-2.17)	1.10 (0.86-1.40)	0.96 (0.42-2.17)	1.10 (0.70-1.72)
		n	3	3	3	3	3	3
	U	EE (95%CI)	0.71 (0.63-0.80)	0.56 (0.43-0.75)	0.45 (0.36-0.55)	0.92 (0.80-1.05)	0.56 (0.42-0.75)	0.65 (0.55-0.77)
		n	1	3	1	1	3	3
All estimates	U	EE (95%CI)	1.04 (0.83-1.29)	0.79 (0.51-1.22)	0.90 (0.46-1.78)	1.13 (0.97-1.31)	0.80 (0.53-1.22)	0.89 (0.74-1.08)
		n	5	7	5	5	7	7
Population = tested for COVID-19[4]								
Best-adjusted	All	EE (95%CI)	0.71 (0.60-0.84)	0.61 (0.52-0.71)	0.52 (0.43-0.64)	0.97 (0.86-1.09)	0.55 (0.47-0.65)	0.70 (0.63-0.77)
		n	10	13	8	8	17	17
	C	EE (95%CI)	0.77 (0.56-1.05)	0.60 (0.45-0.79)	0.53 (0.34-0.83)	0.90 (0.76-1.08)	0.58 (0.43-0.80)	0.77 (0.64-0.92)
		n	4	5	4	4	5	5
	U	EE (95%CI)	0.67 (0.52-0.86)	0.62 (0.51-0.75)	0.49 (0.42-0.58)	1.04 (0.85-1.28)	0.55 (0.45-0.67)	0.67 (0.58-0.78)
		n	6	8	4	4	11	11
All estimates	U	EE (95%CI)	0.72 (0.62-0.85)	0.61 (0.50-0.73)	0.53 (0.46-0.61)	0.97 (0.87-1.09)	0.55 (0.46-0.66)	0.71 (0.64-0.79)
		n	10	12	8	8	17	17

[1]Best-adjusted = estimates for a study selected in preference order of adjustment (C = comorbidities, D = demographics only, U = unadjusted, P = includes post-coronavirus disease responses).
[2]EE: Effect estimate from random-effects meta-analysis.
[3]For population = general, the results are from six studies, one providing sex-specific estimates. Four provided odds ratio (OR)s, one hazard ratios and one relative risk (RR)s and ORs.
[4]For population = tested, the results are from 15 studies, two providing sex-specific estimates, with 13 providing only ORs and two RRs, one of these also providing ORs. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

COVID-19. While the unadjusted estimates show increased hospitalization rates in former and ever smokers, those adjusted for comorbidities show no indication of an increase for any index of smoking.

Supplementary Table 3 shows additional results from three studies. BIOBNK provided results based on the general population, GDEM and GU provided results on those tested for COVID-19, and GU provided results based on those hospitalized and unmatched controls. The results are rather conflicting, with GU showing markedly lower hospitalization rates in current smokers, and the other studies increased rates. In BIOBNK, adjustment tended to reduce the associations, although they remained statistically significant. GDEM reported only results adjusted for demographics and GU reported only unadjusted results.

Endpoint: Admitted to ICU

Table 3 shows the meta-analysis results based on estimates from eight studies of those positive for COVID-19 and 14 of those hospitalized with COVID-19. Supplementary Table 4 shows the results from one general population study, and one study comparing intensive care patients with unmatched controls. Most estimates considered in Table 3 are unadjusted, not even for age, and show little evidence of an

Table 2 Main results for endpoint: Hospitalized with coronavirus disease 2019

Selection of estimates[1]		Statistic[2]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Population = positive for COVID-19[3]								
Best-adjusted	All	EE (95%CI)	1.34 (1.13-1.60)	1.00 (0.86-1.16)	1.05 (0.84-1.32)	1.31 (0.93-1.85)	1.31 (1.12-1.54)	1.38 (1.17-1.63)
		n	19	13	11	11	22	22
	C	EE (95%CI)	1.00 (0.76-1.32)	0.90 (0.76-1.07)	0.82 (0.52-1.30)	0.87 (0.67-1.12)	0.96 (0.79-1.18)	0.93 (0.80-1.08)
		n	6	6	4	4	8	8
	U	EE (95%CI)	1.53 (1.14-2.06)	1.03 (0.75-1.41)	1.13 (0.81-1.59)	1.77 (1.04-3.04)	1.44 (1.03-2.01)	1.71 (1.24-2.36)
		n	10	6	6	6	11	11
All estimates	U	EE (95%CI)	1.64 (1.41-1.91)	0.97 (0.71-1.32)	1.20 (1.01-1.42)	1.75 (1.35-2.27)	1.47 (1.21-1.77)	1.72 (1.46-2.02)
		n	18	13	11	11	21	21

[1]Best-adjusted = estimates for a study selected in preference order of adjustment (C = comorbidities, D = demographics only, U = unadjusted, P = includes post-COVID responses).
[2]EE = effect estimate from random-effects meta-analysis.
[3]The results are from 21 studies, one study providing sex-specific estimates. Nineteen studies provide odds ratio (OR)s, one relative risk (RR)s and hazard ratios, and one ORs and RRs. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

association between smoking and ICU admission. Exceptionally, the data in Supplementary Table 4 show reduced admission rates in current smokers, and increased admission rates in former smokers, tending to diminish and become marginally significant after adjustment in the HIPPIS study.

Endpoint: Mechanically ventilated

Fourteen studies provided results where the population involved patients hospitalized with COVID-19. While the best-adjusted effect estimates in Table 4 were greater than 1.0 for each smoking index, none were statistically significant at $P < 0.05$.

Supplementary Table 5 summarizes the results from three studies where the population was those tested for COVID-19. VETERA, which provided the most detailed results, did not demonstrate any clear association, with estimates for ever and for former smoking significantly increased when unadjusted, but close to 1.0 and non-significant when adjusted for comorbidities. In ESSVRD, a significant unadjusted increase again was non-significant after adjustment for comorbidities. Exceptionally, MUNOZP reported a very high OR for ever smoking after adjustment for comorbidities.

Endpoint: Severe COVID-19

Table 5 shows the meta-analysis results from five studies on those positive for COVID-19 and 10 studies on those hospitalized with COVID-19. As shown in Table 5, definitions of severity varied by study. Few effect estimates were adjusted for comorbidities. The smoking indices were generally associated with a small increase in severity, but this was only significant at $P<0.05$ in one of the 12 best-adjusted meta-analysis estimates.

Endpoint: Died

Table 6 summarizes the results using data from 25 studies of those hospitalized with COVID-19, and eight studies of those positive for COVID-19 regardless of hospitalization. The estimates adjusted for comorbidities were virtually never statistically significant and usually close to 1.00, but the unadjusted estimates were nearly always elevated and often statistically significant. This was very clearly illustrated by the results for "closest to ever smoking" where about a two-fold increase was seen for the unadjusted results, with little or no increase seen for the comorbidity adjusted results, regardless of the population studied. It is also clear that higher unadjusted estimates were seen for former or ever smoking than for current smoking, perhaps because

Table 3 Main results for endpoint: Admitted to intensive care unit

Selection of estimates[1]		Statistic[2]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Population = positive for COVID-19[3]								
Best-adjusted	All	EE (95%CI)	1.57 (1.14-2.17)	0.87 (0.67-1.12)	0.94 (0.60-1.48)	1.55 (1.00-2.42)	1.13 (0.82-1.54)	1.40 (1.09-1.80)
		n	7	6	5	5	8	8
	C	EE (95%CI)	1.68 (1.15-2.46)	0.85 (0.77-0.94)	0.61 (0.11-3.36)	1.58 (0.97-2.57)	1.14 (0.54-2.41)	1.30 (0.74-2.31)
		n	2	2	1	1	3	3
	U	EE (95%CI)	1.42 (0.94-2.14)	0.94 (0.57-1.57)	0.98 (0.60-1.60)	1.55 (0.91-2.65)	0.98 (0.60-1.60)	1.42(0.94-2.14)
		n	4	4	4	4	4	4
All estimates	U	EE (95%CI)	1.52 (1.04-2.22)	1.03 (0.56-1.88)	0.92 (0.60-1.43)	1.68 (1.05-2.68)	1.11 (0.62-2.00)	1.61 (1.13-2.29)
		n	5	6	5	5	6	6
Population = hospitalized with COVID-19[4]								
Best-adjusted	All	EE (95%CI)	1.10 (0.99-1.22)	0.90 (0.72-1.12)	0.88 (0.72-1.08)	1.21 (0.99-1.47)	1.02 (0.87-1.21)	1.11 (0.98-1.25)
		n	9	9	6	6	14	14
	C	EE (95%CI)	1.01 (0.88-1.16)	0.80 (0.61-1.04)	0.81 (0.61-1.07)	1.07 (0.92-1.24)	0.81 (0.61-1.07)	1.01 (0.88-1.16)
		n	1	1	1	1	1	1
	U	EE (95%CI)	1.22 (1.04-1.43)	1.09 (0.93-1.27)	0.96 (0.70-1.31)	1.30 (1.00-1.70)	1.16 (1.05-1.28)	1.19 (1.08-1.30)
		n	8	7	5	5	12	12
All estimates	U	EE (95%CI)	1.20 (1.09-1.32)	1.00 (0.87-1.13)	0.89 (0.74-1.06)	1.28 (1.10-1.48)	1.05 (0.95-1.17)	1.14 (1.04-1.24)
		n	9	9	6	6	14	14

[1]Best-adjusted = estimates for a study selected in preference order of adjustment (C = comorbidities, U = unadjusted, P = includes post coronavirus disease responses).
[2]EE = effect estimate from random-effects meta-analysis.
[3]For population = positive, the results are from eight studies. Six provided odds ratio (OR)s and two relative risk (RR)s;
[4]For population = hospitalized, the results are from 14 studies. Twelve provided ORs and two RRs. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

former smokers tend to be older than current or never smokers.

Table 6 does not include results where the population studied was those admitted to the ICU, as these form a subset of those reported in Table 7; see the next section.

Results were also available from three studies based on other populations (Supplementary Table 6). OPENSA provided estimates based on the general population, and these seem consistent with the pattern shown in Table 6. For former smoking, for example, an unadjusted estimate of 2.53 [95% confidence interval (CI): 2.43-2.63] reduced to 1.19 (1.14-1.24) after adjustment for comorbidities. Two other studies only reported unadjusted results. PELLAU found no increase in smokers (undefined) when comparing hospitalized patients with and without COVID-19, while GU reported an increased risk of death in former and ever smokers and a decreased risk in current smokers, whether the tested population was considered or whether decedents were compared to unmatched controls.

Not considered above was the KNIGHT study, which provided no effect estimates, merely stating that ever cigarette smoking predicted death from COVID-19.

Other endpoints

As shown in Table 7, nine studies reported results on the endpoint worsened or died, based on those with severe disease. Most estimates relate to death among those in the

Table 4 Main results for endpoint: Mechanically ventilated

Selection of estimates [1]		Statistic [2]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Population = hospitalized for COVID-19 [3]								
Best-adjusted	All	EE (95%CI)	1.05 (0.88-1.25)	1.11 (0.72-1.71)	1.12 (0.60-2.09)	1.16 (0.87-1.55)	1.08 (0.86-1.36)	1.11 (0.92-1.35)
		n	12	8	5	5	15	15
	C	EE (95%CI)	1.04 (0.79-1.36)	1.94 (0.40-9.49)	2.77 (0.28-27.2)	2.43 (0.32-18.5)	0.99 (0.77-1.29)	1.04 (0.79-1.36)
		n	7	2	2	2	7	7
	U	EE (95%CI)	1.06 (0.94-1.21)	1.12 (0.60-2.09)	0.76 (0.59-0.98)	1.10 (0.96-1.27)	1.16 (0.80-1.67)	1.24 (0.99-1.54)
		n	5	5	3	3	7	7
All estimates	U	EE (95%CI)	1.07 (0.92-1.26)	1.10 (0.80-1.51)	1.09 (0.60-1.95)	1.12 (0.98-1.28)	1.12 (0.98-1.38)	1.17 (1.00-1.37)
		n	10	8	5	5	13	13

[1]Best-adjusted = estimates for a study selected in preference order of adjustment (C = comorbidities, D = demographics only, U = unadjusted, P = includes post-coronavirus disease responses).
[2]EE = effect estimate from random-effects meta-analysis.
[3]Fourteen studies provided results, one providing estimates for two age groups. Thirteen studies provided odds ratios and one relative risks. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

ICU or those requiring mechanical ventilation. There was a tendency for smoking to be positively associated with the endpoint. However, each estimate was unadjusted even for demographic variables.

Two studies reported results for endpoints worse than hospitalization among those tested for COVID-19 (see Supplementary Table 7). Based on estimates adjusted for demographics only, GDEM reported a significant increased risk of pneumonia in current smokers, but no increase in intensive care admissions or need for mechanical ventilation. Similar to the results shown in Supplementary Table 6 for death, and based on unadjusted estimates, GU reported an increased risk of ICU admission in former and ever smokers and a decreased risk in current smokers.

Consistency of results in subgroups

Table 8 compares best-adjusted effect estimates by level of adjustment, effect estimate type, location and study size separately for the indices of smoking closest to current smoking and closest to ever smoking, and for the six combinations of endpoint and population where data were available for at least 10 studies.

For level of adjustment, the results echo those summarized above, with adjustment for comorbidities eliminating unadjusted associations of both current and ever smoking with hospitalization within those COVID-19 positive, and with ICU admission and death within those hospitalized for COVID-19.

Significant variation by type of effect estimate was only seen in two of the 12 analyses, and where it was seen may reflect the fact that the unadjusted estimates were typically ORs.

There is no convincing evidence that effect estimates vary by location.

Large studies, involving 50000 or more individuals, showed no significant increases with smoking in any of the analyses shown. Again, higher effect estimates seen in smaller studies may reflect a greater tendency for such studies to report unadjusted results.

Although meta-analyses were attempted by sex, none of them included sex-specific results from more than two studies, and the results are not shown in Table 8. There were even fewer studies reporting results by age, or by sex and age jointly, so meta-analyses by these factors were not attempted.

Dose-response results

Only one study, HIPPIS, reported results by amount smoked in current smokers, and only one, TOOLKI, by quit duration in former smokers. Neither study showed a

Table 5 Results for endpoint: Severe coronavirus disease 2019[1]

Selection of estimates[2]		Statistic[3]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Population = positive[4]								
Best-adjusted	All	EE (95%CI)	1.39 (1.07-1.80)	1.04 (0.91-1.20)	1.27 (0.82-1.95)	1.20 (0.62-2.32)	1.10 (0.97-1.25)	1.10 (0.97-1.25)
		n	7	5	4	4	8	8
	C	EE (95%CI)	-	1.02 (0.88-1.18)	-	-	1.02 (0.88-1.18)	1.02 (0.88-1.18)
		n	0	1	0	0	1	1
	U	EE (95%CI)	1.30 (0.94-1.79)	1.26 (0.82-1.93)	1.27 (0.82-1.95)	1.20 (0.62-2.32)	1.35 (0.95-1.92)	1.30 (0.94-1.79)
		n	5	4	4	4	5	5
All estimates	U	EE (95%CI)	1.63 (1.27-2.10)	1.17 (1.09-1.25)	1.27 (0.82-1.95)	1.20 (0.62-2.32)	1.48 (1.13-1.94)	1.45 (1.12-1.86)
		n	7	5	4	4	8	8
Population = hospitalized[5]								
Best-adjusted	All	EE (95%CI)	1.15 (0.87-1.51)	1.05 (0.93-1.18)	0.74 (0.49-1.12)	1.07 (0.87-1.32)	1.08 (0.94-1.25)	1.09 (0.98-1.22)
		n	7	5	2	2	10	10
	C	EE (95%CI)	1.35 (0.66-2.75)	0.76 (0.50-1.15)	0.77 (0.51-1.17)	1.06 (0.86-1.31)	1.23 (0.46-3.27)	1.35 (0.66-2.75)
		n	2	1	1	1	2	2
	U	EE (95%CI)	1.38 (1.04-1.83)	1.12 (1.04-1.21)	0.29 (0.03-2.64)	1.25 (0.56-2.76)	1.17 (0.99-1.37)	1.14 (1.06-1.23)
		n	4	3	1	1	6	6
All estimates	U	EE (95%CI)	1.40 (1.20-1.63)	1.14 (1.04-1.24)	0.89 (0.54-1.46)	1.48 (1.23-1.80)	1.17 (1.08-1.27)	1.21 (1.12-1.29)
		n	6	5	2	2	9	9

[1]In most studies the endpoint was severe coronavirus disease 2019 (COVID-19) as defined by the author, but it was admitted to ICU or died in two studies, pneumonia in one, increased oxygen required in one and COVID-19 progressed in one.
[2]Best-adjusted = estimates for a study selected in preference order of adjustment (C = comorbidities, U = unadjusted, P = includes post-COVID responses).
[3]EE = effect estimate from random-effects meta-analysis.
[4]For population = positive, the results are from five studies, one study providing four estimates by age and sex. All estimates are odds ratio (OR)s.
[5]For population = hospitalized, the results are from 10 studies, nine providing ORs and one relative risks and hazard ratios. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

significant dose-response relationship for any of the endpoint/population combinations considered (results not shown).

DISCUSSION

Table 9 summarizes the results from the meta-analyses of the 10 endpoint/population combinations shown in Tables 1 to 6. Results for the best estimates are shown, where estimates adjusted for comorbidities (and not responses to COVID-19) were preferred, with those adjusted for demographics preferred to unadjusted estimates, and those adjusted for factors including responses to the infection being least preferred. Results are also presented for comorbidity-adjusted estimates and for unadjusted estimates. The results show no consistent evidence of publication bias.

The clearest result shows that, of those tested for COVID-19, smoking was associated with a reduced risk of positivity (Figures 2 and 3), with less clear evidence of a negative association between smoking and positivity seen among the general population.

Table 6 Main results for endpoint: Died

Selection of estimates[1]		Statistic[2]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Population = hospitalized with COVID-19[3]								
Best-adjusted	All	EE (95%CI)	1.42 (1.19-1.70)	0.99 (0.89-1.10)	1.12 (0.98-1.29)	1.61 (1.34-1.92)	1.27 (1.08-1.49)	1.42 (1.22-1.65)
		n	18	10	10	10	25	25
	C	EE (95%CI)	1.07 (0.82-1.38)	0.64 (0.12-3.35)	1.02 (0.70-1.49)	1.15 (1.02-1.29)	1.09 (0.84-1.40)	1.12 (0.91-1.39)
		n	8	2	3	3	10	10
	U	EE (95%CI)	1.78 (1.40-2.26)	0.95 (0.77-1.16)	1.18 (0.91-1.52)	1.92 (1.48-2.50)	1.52 (1.22-1.91)	1.79 (1.47-2.19)
		n	9	6	6	6	13	13
All estimates	U	EE (95%CI)	1.59 (1.37-1.83)	1.00 (0.88-1.13)	1.12 (0.97-1.29)	1.80 (1.48-2.17)	1.33 (1.18-1.51)	1.58 (1.39-1.78)
		n	15	10	9	9	20	20
Population = with COVID-19[4]								
Best-adjusted	All	EE (95%CI)	1.41 (0.93-2.14)	0.96 (0.92-1.01)	0.98 (0.72-1.32)	1.61 (0.79-3.29)	1.08 (0.82-1.42)	1.34 (0.96-1.86)
		n	7	6	5	5	8	8
	C	EE (95%CI)	0.99 (0.87-1.13)	0.96 (0.92-1.01)	0.85 (0.67-1.07)	1.04 (0.91-1.19)	0.96 (0.92-1.01)	0.97 (0.93-1.02)
		n	3	4	3	3	4	4
	U	EE (95%CI)	2.07 (1.12-3.82)	0.93 (0.60-1.46)	1.49 (0.94-2.36)	2.70 (1.11-6.58)	1.59 (1.40-1.80)	2.07 (1.12-3.82)
		n	3	2	2	2	3	3
All estimates	U	EE (95%CI)	2.04 (1.39-3.02)	1.06 (0.39-2.90)	1.07 (0.72-1.58)	2.58 (1.31-5.07)	1.52 (0.89-2.60)	2.22 (1.51-3.27)
		n	5	5	4	4	6	6

[1]Best-adjusted = estimates for a study selected in preference order of adjustment [C = comorbidities, D = demographics only, U = unadjusted, P = includes post-coronavirus disease (COVID) responses.

[2]EE = effect estimate from random-effects meta-analysis.

[3]For population = hospitalized, the results are from twenty four studies, one providing age-specific estimates. Seventeen provided only odds ratio (OR)s, two only hazard ratio (HR)s, two only relative risk (RR)s, and three RRs and HRs, one of these also providing ORs.

[4]For population = with COVID-19, the results are from eight studies, six providing ORs, one RRs and one HRs and RRs. COVID-19: Coronavirus disease 2019; CI: Confidence interval.

In contrast, all the best estimates for the other eight endpoint/population combinations, each of which relate to adverse events in those positive for, or hospitalized with COVID-19, were greater than 1 (ranging from 1.02 to 1.42), with five of the 16 estimates statistically significant (at $P < 0.05$). However, there was a clear difference between estimates unadjusted for other risk factors, where nine of the 16 estimates were significant, and those adjusted for comorbidities where none were significant and six were below 1.0. This difference is strikingly seen for the most commonly considered endpoint/population combination - died among those hospitalized - where (Figures 4 and 5) unadjusted estimates of 1.52 (95%CI: 1.22-1.91) for the smoking index closest to current smoking and 1.79 (1.47-2.19) for that closest to ever smoking can be contrasted with comorbidity adjusted estimates of, respectively, 1.09 (0.84-1.40) and 1.12 (0.91-1.39).

A major limitation of the available data is that, of the 73 studies which provided effect estimates, adjusted results were available for only 42. In most epidemiological contexts, effect estimates adjusted for age and sex are a basic starting point for analysis, but this was not so here. Given the different age distribution of current, former and never smokers and the strong age relationship to severe COVID-19 and death, unadjusted estimates would seem likely to be biased, as would the analyses

Table 7 Results for endpoint: Worsened or died[1]

Selection of estimates[2]	Statistic[3]	Ever *vs* never	Current *vs* non-current	Current *vs* never	Former *vs* never	Closest to current	Closest to ever
Unadjusted[4]	EE (95%CI)	1.34 (1.00-1.79)	1.31 (1.06-1.62)	1.21 (0.35-4.17)	2.01 (0.67-6.09)	1.27 (1.15-1.39)	1.36 (1.15-1.62)
	n	5	5	3	3	9	9

[1]Seven studies considered death among those admitted to the intensive care unit (ICU), and one of these and one other considered death among those mechanically ventilated. One considered admission to ICU or death among those hospitalized with severe coronavirus disease 2019.
[2]All estimates were unadjusted.
[3]EE = effect estimate from random-effects meta-analysis.
[4]The data are from nine studies, five reporting odds ratio (OR)s, two relative risk (RR)s and two RRs and hazard ratios, one of these also reporting ORs. CI: Confidence interval.

which adjusted for variables representing a response to the virus. The most useful analyses were based on estimates adjusted for demographics only, or those adjusted also for comorbidities. These answer different questions. Analyses adjusted for demographics and comorbidities attempted to answer the question "Is a smoker more at risk of COVID-19 related outcome (such as hospitalization, admission to intensive care, undergoing mechanical ventilation or death) than an otherwise equivalent never smoker of the same age, sex and other relevant demographics and health status pre-pandemic?" This is a valid question and is somewhat equivalent to that investigated in a cohort study where smoking, demographics and health status were recorded at baseline, and smoking was related to an outcome occurring during follow-up. In analyses adjusting for demographics only, any increased risk of COVID-19 related outcomes in smokers may be due to their poorer status of health pre-pandemic. While clear answers to both questions would be nice to have, it must be noted that there are very few studies providing effect estimates adjusted only for demographics. Thus, considering deaths in the hospitalized population, only three out of 25 studies with relevant data provided estimates adjusted for demographics only, and none provided comparable unadjusted, demographic adjusted and comorbidity adjusted effect estimates.

Another problem is that some studies provided ORs, some RRs and some HRs. Where results for the relevant 2 × 2 table on exposure × outcome were available, and the ORs and RRs were both estimable, we generally used the OR, using the RR only where the source paper had reported adjusted RRs or HRs. Although we could have used an alternative strategy, it is doubtful whether this would have materially affected our results, given the general consistency of the results by type of effect estimate.

Another possible concern is with the mortality data. Much relates to deaths occurring in patients hospitalized with COVID-19, and many publications implicitly assume all those deaths were due to the virus, when some might have been due to other causes. However, given this proportion is small, it seems probable that this would only result in a minor bias.

More concern relates to the quality of the smoking data. There are two issues here. One is the way smoking was recorded and defined, with eight studies reporting results for smoking undefined, and only 36 distinguishing current and former smoking. Also, only one study reported results by amount smoked, and only one reported results by duration of quit. Furthermore, papers generally did not provide details on the smoking questions asked, or when they were asked.

The other issue is that many studies derived their smoking data from medical records, known to be incomplete and inaccurate[37-39]. While many studies gave no information concerning missing data on smoking, 35 did so, and in 13 the proportion with missing data exceeded 20%, giving concern about the validity of their effect estimates. It would not surprise us to find that, in some studies that did not mention missing data, the "non-smokers" included some individuals never actually asked about their smoking.

Recent publications, particularly by Farsalinos *et al*[2-4,6-8], have observed that the prevalence of smoking seen in the studies of hospitalized patients was substantially less than reported in national statistics by a factor of four or so, and have suggested that smokers might be protected against getting COVID-19. While the mean percentage of current and former smokers in the studies of hospitalized patients that we considered (current 7.76%, SE 1.00%; ever 33.24%, SE 2.01%) was clearly less than in the studies of the general population (current 15.14%, SE 1.34%; ever 44.0%, SE

Table 8 Variations in best-adjusted effect estimates by level of adjustment, effect estimate type, location and study size

Factor/level	Endpoint	CP	H	IC	MV	S	M
	Population	T	CP	H	H	H	H
	Estimates	17	22	14	15	10	25
Estimates for closest to current smoking							
Adjustment	U	0.55 (0.45-0.67)	1.44 (1.03-2.01)	1.16 (1.05-1.28)	1.16 (0.80-1.67)	1.17 (0.99-1.37)	1.52 (1.22-1.91)
	D	0.23 (0.09-0.59)	1.52 (1.18-1.95)	0.73 (0.56-0.96)	0.85 (0.67-1.07)	1.02 (0.89-1.16)	1.01 (0.87-1.17)
	C	0.58 (0.43-0.80)	0.96 (0.79-1.18)	0.81 (0.61-1.07)	0.99 (0.77-1.29)	1.23 (0.46-3.27)	1.09 (0.84-1.40)
	PC	-	-	-	-	0.71 (0.41-1.23)	-
	P	NS	< 0.05	< 0.001	NS	NS	< 0.05
Estimate type	OR	0.54 (0.46-0.63)	1.26 (0.97-1.64)	1.07 (0.87-1.32)	1.13 (0.88-1.44)	1.09 (0.93-1.27)	1.35 (1.07-1.70)
	RR	0.64 (0.31-1.34)	1.25 (1.20-1.31)	0.90 (0.71-1.15)	0.73 (0.56-0.95)	-	1.11 (0.87-1.40)
	HR	-	1.10 (0.97-1.24)	-	-	1.02 (0.64-1.63)	1.15 (0.89-1.50)
	P	NS	NS	NS	< 0.05	NS	NS
Location	United States	0.45 (0.37-0.54)	1.27 (1.11-1.46)	1.00 (0.81-1.22)	1.02 (0.83-1.26)	1.06 (0.72-1.56)	1.21 (0.95-1.53)
	Other	0.64 (0.55-0.73)	1.11 (0.48-2.57)	1.08 (0.79-1.49)	1.05 (0.65-1.71)	1.10 (1.02-1.17)	1.34 (1.07-1.69)
	P	< 0.01	NS	NS	NS	NS	NS
Study size	< 500	-	0.99 (0.58-1.69)	1.25 (0.91-1.71)	1.35 (1.02-1.80)	1.02 (0.65-1.59)	1.29 (0.97-1.72)
	500-	0.40 (0.27-0.61)	1.42 (1.17-1.72)	1.07 (0.69-1.65)	0.92 (0.34-2.46)	1.62 (1.14-2.29)	1.57 (1.20-2.05)
	5000-	0.51 (0.39-0.66)	1.43 (0.97-2.10)	0.64 (0.29-1.42)	0.83 (0.70-0.98)	0.81 (0.56-1.19)	1.03 (0.83-1.28)
	50,000+	0.67 (0.51-0.87)	1.00 (0.85-1.18)	0.97 (0.72-1.31)	0.98 (0.61-1.59)	1.09 (0.99-1.19)	0.94 (0.82-1.07)
	P	NS	< 0.05	NS	< 0.05	NS	< 0.01
Estimates for closest to ever smoking							
Adjustment	U	0.67 (0.58-0.78)	1.71 (1.24-2.36)	1.19 (1.08-1.30)	1.24 (0.99-1.54)	1.14 (1.06-1.23)	1.79 (1.47-2.19)
	D	0.23 (0.09-0.59)	1.41 (1.04-1.92)	0.73 (0.56-0.96)	0.85 (0.67-1.07)	1.02 (0.89-1.16)	1.11 (0.81-1.53)
	C	0.77 (0.64-0.92)	0.93 (0.80-1.08)	1.01 (0.88-1.16)	1.04 (0.79-1.36)	1.35 (0.66-2.75)	1.12 (0.91-1.39)
	PC	-	-	-	-	0.71 (0.41-1.23)	-
	P	< 0.05	< 0.001	< 0.01	NS	NS	< 0.01
Estimate type	OR	0.67 (0.59-0.76)	1.39 (1.08-1.79)	1.16 (0.99-1.37)	1.14 (0.91-1.42)	1.10 (0.98-1.24)	1.53 (1.19-1.96)
	RR	0.87 (0.59-1.27)	1.25 (1.20-1.31)	1.01 (0.89-1.15)	1.02 (0.88-1.18)	-	1.16 (1.05-1.29)
	HR	-	1.03 (0.95-1.11)	-	-	1.02 (0.64-1.63)	1.28 (1.14-1.44)
	P	NS	< 0.001	NS	NS	NS	NS
Location	United States	0.65 (0.50-0.84)	1.38 (1.14-1.67)	1.10 (0.99-1.23)	1.08 (0.90-1.30)	1.15 (0.87-1.51)	1.43 (1.11-1.84)
	Other	0.74 (0.66-0.82)	1.31 (0.64-2.71)	1.08 (0.79-1.49)	1.05 (0.65-1.71)	1.10 (1.02-1.17)	1.43 (1.19-1.73)
	P	NS	NS	NS	NS	NS	NS
Study size	< 500	-	1.21 (0.84-1.75)	1.25 (0.93-1.69)	1.29 (0.95-1.76)	1.06 (0.74-1.52)	1.36 (1.05-1.75)
	500-	0.55 (0.37-0.80)	1.32 (0.99-1.76)	1.04 (0.92-1.18)	1.24 (0.77-2.00)	1.62 (1.14-2.29)	1.70 (1.34-2.16)
	5000-	0.64 (0.50-0.81)	1.71 (1.18-2.49)	1.48 (1.10-2.00)	0.83 (0.70-0.98)	1.00 (0.83-1.22)	1.27 (0.96-1.68)
	50000+	0.81 (0.73-0.89)	0.97 (0.88-1.07)	0.97 (0.73-1.28)	1.10 (0.79-1.52)	1.09 (0.99-1.19)	0.94 (0.82-1.07)
	P	< 0.05	< 0.01	NS	< 0.05	NS	< 0.001

The table shows effect estimates with 95% confidence interval. Variation between levels of a factor is coded as $P < 0.001$, $P < 0.01$, $P < 0.05$ or NS ($P \geq 0.05$). C: Adjusted for comorbidities; CP: Coronavirus disease 2019 positive; D: Adjusted for demographics only; H: Hospitalized; HR: Hazard ratio; IC: Intensive

care; M: Mortality; MV: Mechanical ventilation; OR: Odds ratio; PC: Adjusted for variables including post-coronavirus disease 2019 responses; RR: Relative risk; S: Severe coronavirus disease 2019; T: Tested for coronavirus disease 2019; U: Unadjusted.

Table 9 Summary of results from meta-analyses[1]

Endpoint	Population	Smoking index = closest to current smoking			Smoking index = closest to ever smoking		
		Best estimate[2]	Comorbidity adjusted[3]	Unadjusted[4]	Best estimate[2]	Comorbidity adjusted[3]	Unadjusted[4]
Positive	General	0.78 (0.57-1.06)[5]	0.96 (0.42-2.17)	0.56 (0.42-0.75)[5]	0.89 (0.73-1.09)	1.10 (0.70-1.72)	0.65 (0.55-0.77)
Positive	Tested	0.55 (0.47-0.65)	0.58 (0.43-0.80)	0.55 (0.45-0.67)	0.70 (0.63-0.77)	0.77 (0.64-0.92)	0.67 (0.58-0.78)
Hospitalized	Positive	1.31 (1.12-1.54)	0.96 (0.79-1.18)	1.44 (1.03-2.01)	1.38 (1.17-1.63)	0.93 (0.80-1.08)	1.71 (1.24-2.36)
ICU admission	Positive	1.13 (0.82-1.54)	1.14 (0.54-2.41)	0.98 (0.60-1.60)	1.40 (1.09-1.80)[5]	1.30 (0.74-2.31)	1.42 (0.94-2.14)
ICU admission	Hospitalized	1.02 (0.87-1.21)	0.81 (0.61-1.07)	1.16 (1.05-1.28)	1.11 (0.98-1.25)	1.01 (0.88-1.16)	1.19 (1.08-1.30)
Mechanically ventilated	Hospitalized	1.08 (0.86-1.36)	0.99 (0.77-1.29)[5]	1.16 (0.80-1.67)	1.11 (0.92-1.35)	1.04 (0.79-1.36)[5]	1.24 (0.99-1.54)
Severe	Positive	1.10 (0.97-1.25)[5]	1.02 (0.88-1.18)	1.35 (0.95-1.92)	1.10 (0.97-1.25)[5]	1.02 (0.88-1.18)	1.30 (0.94-1.79)
Severe	Hospitalized	1.08 (0.94-1.25)	1.23 (0.46-3.27)	1.17 (0.99-1.37)	1.09 (0.98-1.22)	1.35 (0.66-2.75)	1.14 (1.06-1.23)
Died	Positive	1.08 (0.82-1.42)	0.96 (0.92-1.01)	1.59 (1.40-1.80)	1.34 (0.96-1.86)	0.97 (0.93-1.02)	2.07 (1.12-3.82)
Died	Hospitalized	1.27 (1.08-1.49)[5]	1.09 (0.84-1.40)	1.52 (1.22-1.91)	1.42 (1.22-1.65)	1.12 (0.91-1.39)	1.79 (1.47-2.19)

[1]See also Tables 1 to 6 for further details. Data shown are random-effects estimates with 95% confidence interval.
[2]Best estimates based on, respectively, 7, 17, 22, 8, 14, 15, 8, 10, 8 and 25 effect estimates (total 134).
[3]Comorbidity adjusted estimates based on, respectively, 3, 5, 8, 3, 1, 7, 1, 2, 4 and 10 (total 44).
[4]Unadjusted estimates based on, respectively, 3, 11, 11, 4, 12, 7, 5, 6, 3 and 13 (total 75). The unadjusted results shown are based only on those included in the best estimates;
[5]Significant publication bias ($P < 0.05$). ICU: Intensive care unit.

1.75%), the difference was only by a factor of 2 or 1.3 rather than about 4. While we also showed a reduced risk in smokers of COVID-19 positivity in those tested (Table 1), the reduction was again much less than the factor reported by Farsalinos *et al* [6]. Although it is possible that some of the difference between our results and those of Farsalinos *et al*[6] has arisen as we excluded Asian studies, while their results mainly came from Asia, the fact that two out of the three studies on the general population that we considered found no reduced risk of hospitalization in smokers (Supplementary Table 3) suggests to us that the low prevalence of smoking seen in hospitalized patients may largely result from incompleteness of data in hospital records, although it would also be consistent with smokers with COVID-19 tending to be less likely to appear for testing or report to hospital.

As noted in the introduction, there were, by the end of September 2020 (the final date of the searches used to produce these results), quite a number of published meta-analyses which relate smoking to adverse events such as deaths or severity of COVID-19[3,6,9-28]. These meta-analyses had various limitations, including little attention to possible data inadequacy, limiting attention to studies of hospitalized patients and considering few, if any, studies conducted outside Asia. They also include paying scant attention to the need for adjusting effect estimates for other risk factors. Many of the studies and meta-analyses we considered demonstrated that dying from COVID-19, for example, was strongly related to various factors associated with smoking, including age, obesity, and a history of respiratory, cardiovascular and other diseases, and yet they attempt to draw conclusions for smoking from unadjusted analyses.

By now, we are aware of further meta-analyses that have been conducted, including those that concentrate on smoking[11,19,29,40,41] and those that consider smoking as one of a list of factors considered[16,20,21,42-55]. While some of the reviews considered far more studies than the earlier reviews, including those in non-Asian populations, the weaknesses seen generally persist. Thus, for example, a recent review

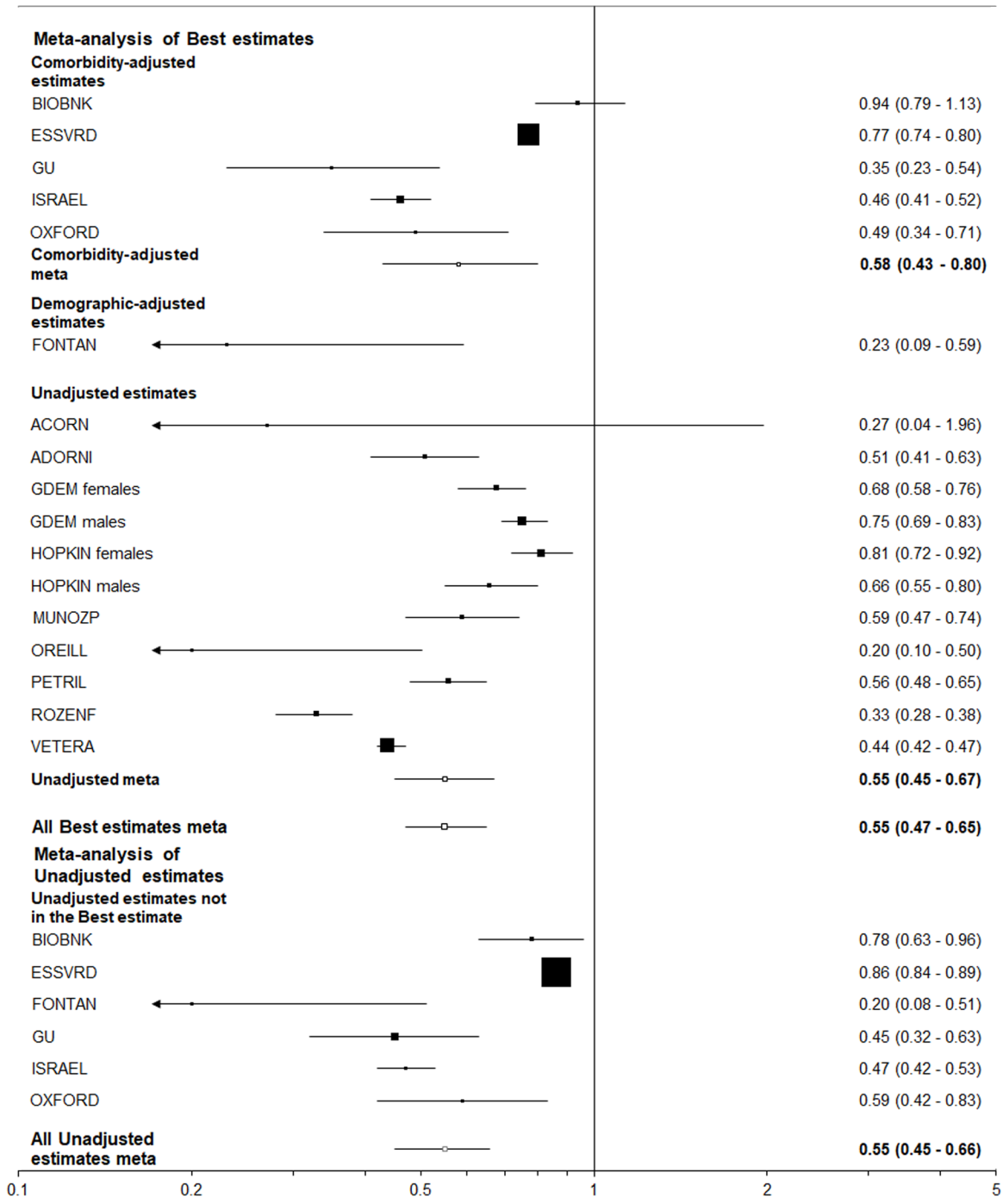


Figure 2 Forest plot of the relationship of the index "closest to current smoking" to positivity for coronavirus disease 2019 among those tested. The individual study effect estimates and random-effects combined effect estimates are shown, both as numbers and as horizontal lines with the weight of the estimate indicated by a box of proportional size. "Best estimates" are selected in the preference order (first to last) comorbidity-adjusted, demographic-adjusted and unadjusted. Estimates from ESSVRD, GDEM and HOPKIN are for current *vs* non-smokers, from FONTAN and OREILL for smokers (undefined) *vs* non-smokers, and from MUNOZP and ROZENF for ever *vs* never smokers, with estimates from the other eight studies being for current *vs* never smokers.

of 109 studies[41] limited attention to hospitalized patients, considered only unadjusted effect estimates, hardly mentioned lack of adjustment as a possible weakness, and paid very limited attention to the possibility that smoking data from hospital records may be inadequate.

That meta-analysis[41] described the 109 studies included as being all of moderate or high quality and some of the other reviews also attempted to evaluate study quality. We did not attempt classification of study quality, but given so many studies used medical records as the source of smoking data and failed to present results adjusted even for basic demographics, we doubt very much that we would have considered more than a few studies to be of high or even moderate quality. This view aligns with that of a recent meta-analysis[29] which rejected 201 of 233 studies as being

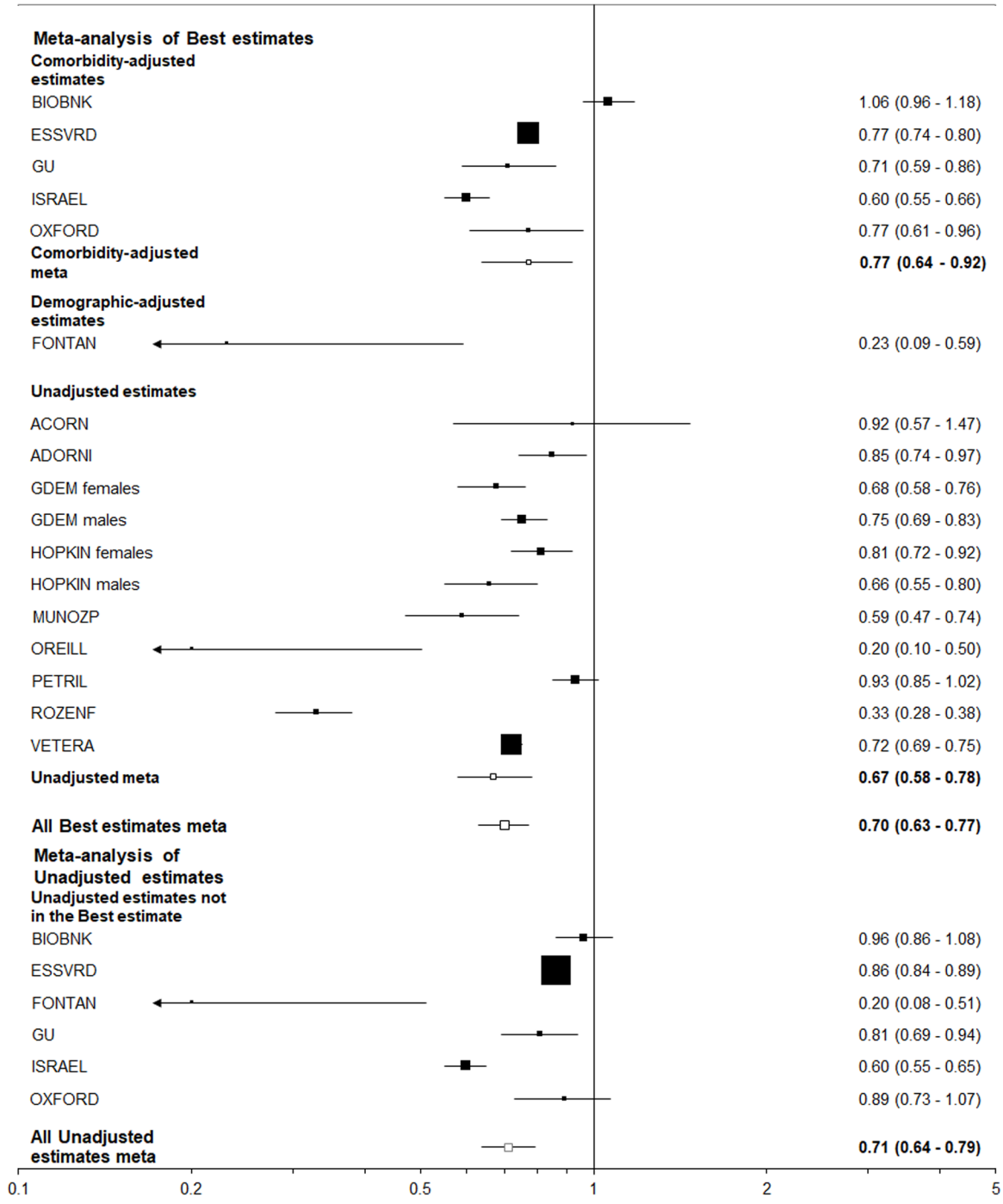


Figure 3 Forest plot of the relationship of the index "closest to ever smoking" to positivity for coronavirus disease 2019 among those tested. This figure is laid out as described for Figure 2. Estimates from ESSVRD, GDEM and HOPKIN are for current *vs* non-smokers, from FONTAN and OREILL for smokers (undefined) *vs* non-smokers, with estimates from the other 10 studies being for ever *vs* never smokers.

of poor quality, with only one of the studies considered in their meta-analyses considered to be of good quality and the rest classified as fair.

Our meta-analyses have various strengths, including giving careful attention to adjustment, considering many combinations of outcome and population at-risk, and including meta-analyses comparing estimates by factors such as location, study size and type of estimate considered. Weaknesses relate mainly to the poor underlying data quality, much from medical records, and many studies failing to provide adjusted estimates. Almost complete lack of data for males and females separately, and by age group is also a limitation, as is the very limited data on amount smoked or quit duration. Other possible limitations relate to the fact that, with the exception of studies from Israel, we did not consider results from other Asian countries; thus, our conclusions may not necessarily apply to all locations. We also did not consider

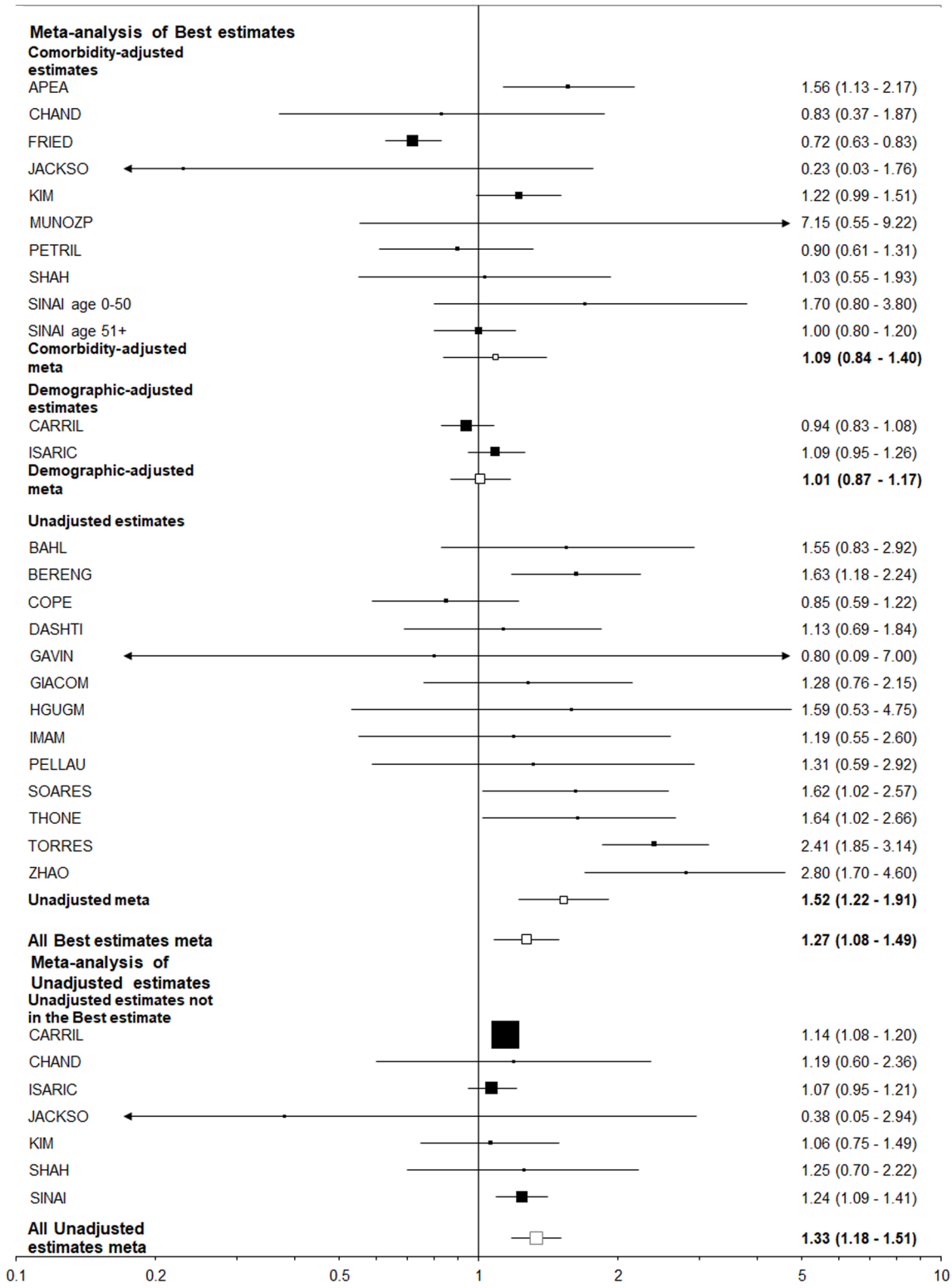


Figure 4 Forest plot of the relationship of the index "closest to current smoking" to death among those hospitalized for coronavirus disease 2019. This figure is laid out as described for Figure 2. Estimates from CARRIL are for current *vs* non-smokers, from APEA, HGUGM, PELLAU, SOARES and TORRES for smokers (undefined) *vs* non-smokers, and from CHAND, FRIED, GIACOM, IMAM, MUNOZP, SHAH, SINAI, THONE and ZHAO for ever *vs* never smokers, with estimates from the other nine studies being for current *vs* never smokers.

studies involving less than 100 cases as their results would be less reliable, and studies of patients with specific diseases as their results would not be generalizable.

However, we feel that our meta-analysis provides a good insight into the relationship between smoking and a variety of endpoints relevant to COVID-19.

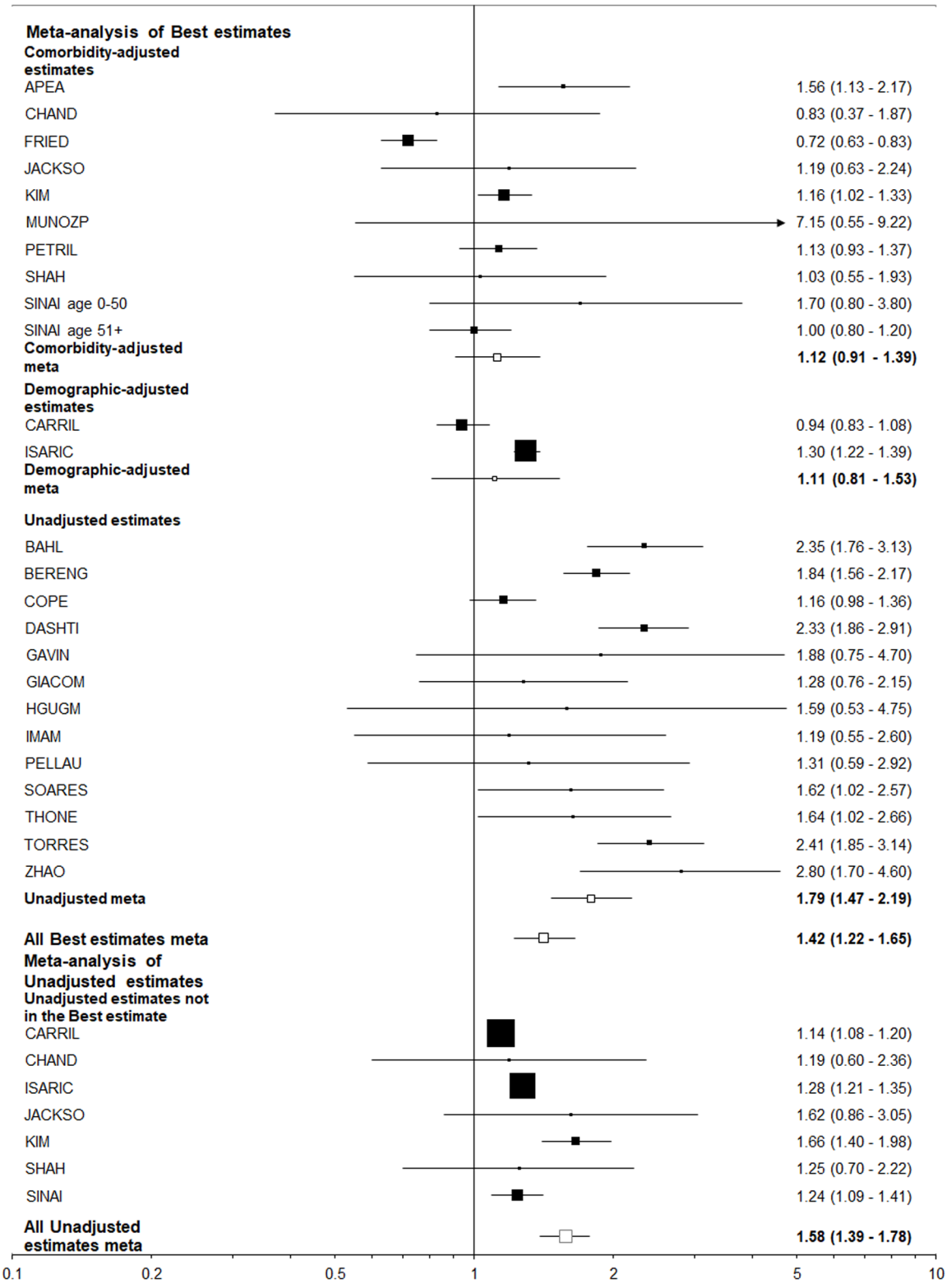


Figure 5 Forest plot of the relationship of the index "closest to ever smoking" to death among those hospitalized for coronavirus disease 2019. This figure is laid out as described for Figure 2. Estimates from CARRIL are for current *vs* non-smokers, from APEA, HGUGM, PELLAU, SOARES and TORRES for smokers (undefined) *vs* non-smokers, and from PETRIL for former *vs* never smokers, with estimates from the other 17 studies being for ever *vs* never smokers.

CONCLUSION

Based on data from 74 studies conducted in Europe, Israel, America and Australasia, many providing only limited results, there is evidence that, among those tested for COVID-19, smokers are less likely to be positive for the virus. There is also less clear evidence of reduced positivity in smokers in the general population. Among those

who are positive for, or hospitalized with, COVID-19 there is a positive association between smoking and both death and severity of COVID-19. This association is most clearly seen for effect estimates unadjusted for other risk factors, and is not evident for estimates adjusted for comorbidities and demographic variables. This suggests that any apparent adverse effect of smoking is due to the poorer prior health status of smokers and that smokers and non-smokers with equivalent demographics and prior health status have a very similar risk of adverse events linked to COVID-19.

ARTICLE HIGHLIGHTS

Research background

Previous meta-analyses relating smoking to coronavirus disease 2019 (COVID-19) are limited by considering few studies, restricting attention to hospitalized patients, giving limited or no attention to the definition of smoking or the reliability of smoking as recorded, and failing to properly consider the effect of adjustment for demographics and comorbidities.

Research motivation

We wished to gain a detailed insight into the effect of smoking on a variety of endpoints in different populations.

Research objectives

To carry out a systematic review, based on epidemiological studies in Europe, Israel, America and Australasia on the relationship of smoking to being tested for COVID-19, being positive for COVID-19, being hospitalized with COVID-19, having severe disease or dying.

Research methods

Literature searches based on publications in English up to September 30, 2020 identified studies of at least 100 individuals, carried out in Europe, Israel, America and Australasia, and unrestricted to those with specific other diseases, and providing information relating smoking to various COVID-related endpoints. Fixed-effect and random-effects meta-analyses were conducted for combinations of index of smoking, endpoint, population and level of adjustment with heterogeneity studied by level of adjustment, study location, and other factors.

Research results

Data were available from 74 studies of highly variable size: 37 in the United States, 10 in the United Kingdom, and up to four elsewhere, with populations most commonly studied being those hospitalized with COVID-19, positive for COVID-19, tested for COVID-19 and the general population. Only 36 studies distinguished current and former smokers, and adjusted results for smoking were only given in 42 studies. Positivity for COVID-19 was reduced among smokers in those tested, but not in the general population. Apparent increases in risk in smokers of hospitalization for COVID-19 among those positive, and of death among those positive and among those hospitalized disappeared following adjustment for pre-existing comorbidities, and there was little evidence of any relationship of smoking with admission to intensive care, being mechanically ventilated or having severe COVID-19, even in the unadjusted results.

Research conclusions

There is some evidence that smoking is associated with a reduced risk of being COVID-19 positive. Any apparent adverse effects of smoking on hospitalization rates among those positive, and on death rates seem due to the poorer prior health status of smokers.

Research perspectives

Evidence from later studies could consolidate these conclusions, and help to explain why, among those tested for COVID-19, current smokers are less likely to be positive.

ACKNOWLEDGEMENTS

We thank Yvonne Cooper for typing various drafts of the paper and obtaining relevant references, and John Fry for comments at various stages.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 August 28; 9(4): 377-388

DOI: 10.13105/wjma.v9.i4.377

ISSN 2308-3840 (online)

META-ANALYSIS

Systematic review and Meta-analysis of efficacy and safety of dienogest in treatment of endometriosis

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Supported by the Science and Technology Department of Henan Province, No. 132300410352.

Conflict-of-interest statement: The authors deny any conflict of interest for this manuscript.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Abstract

BACKGROUND

The quality of life of women with endometriosis is substantially adversely affected by the pelvic pain caused by this disease. However, the choice of medication for endometriosis remains controversial, and no drug has been clearly proven to be superior to others.

AIM

To assess the efficacy and safety of dienogest, a synthetic progestin, in the treatment of women with painful symptoms of endometriosis.

METHODS

PubMed, EMBASE, the Cochrane Library, and the Web of Science databases were searched from their inceptions to January 21, 2020 for randomized controlled trials (RCTs) that compared dienogest with other popular prescription drugs for the treatment of endometriosis. Two reviewers extracted the data. Mean difference (MD) values and risk ratios (RRs) with 95% confidence intervals (CIs) were calculated.

RESULTS

Ultimately, seven RCTs with a total of 1493 participants met the requirements for this review. Dienogest was found to more effective than placebo in alleviating

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Manuscript source: Unsolicited manuscript

Specialty type: Obstetrics and gynecology

Country/Territory of origin: China

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: June 16, 2021
Peer-review started: June 16, 2021
First decision: July 6, 2021
Revised: July 17, 2021
Accepted: August 20, 2021
Article in press: August 20, 2021
Published online: August 28, 2021

P-Reviewer: Rakhshan V
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Ma YJ

endometriosis-related pain (MD = -32.93, 95%CI: -44.63 to -21.23), but led to a more significant decline in plasma estradiol concentrations than placebo (MD = -44.7, 95%CI: -62.24 to -24.69). Dienogest was superior to gonadotropin-releasing hormone analogues (GnRH-a) in relieving pain (MD = -2.41, 95%CI: -3.58 to -1.24). Moreover, compared with dienogest, GnRH-a were significantly more likely to lead to the loss of bone mineral density (MD = 2.77, 95%CI: 0.16 to 5.37) and were significantly associated with a higher incidence of headaches (RR = 0.68, 95%CI: 0.52 to 0.91) and hot flushes (RR = 0.43, 95%CI: 0.18 to 1.02).

CONCLUSION

This meta-analysis demonstrated that dienogest may be a better pain-relief treatment for endometriosis patients, due to its high efficacy and tolerability.

Key Words: Dienogest; Gonadotrophin-releasing hormone analogues; Meta-analysis; Endometriosis; Medication

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Core Tip: We comprehensively and systematically analyzed the safety and effectiveness of dienogest for the treatment of endometriosis-related pain. Only high-quality randomized controlled trials that compared dienogest with other drugs, such as gonadotrophin-releasing hormone analogues and placebo, were included in the meta-analysis. The results provide guidelines for the standardization of the clinical use of medications for endometriosis and would improve the choice of medications for patients.

Citation: Lin SC, Wang XY, Fu XL, Yang WH, Wu H, Bai Y, Shi ZN, Du JP, Wang BJ. Systematic review and Meta-analysis of efficacy and safety of dienogest in treatment of endometriosis. *World J Meta-Anal* 2021; 9(4): 377-388
URL: https://www.wjgnet.com/2308-3840/full/v9/i4/377.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i4.377

INTRODUCTION

Endometriosis is defined as the presence of endometrial glands and stroma outside of the uterine cavity, and affects about 200 million women worldwide[1-3]. Although the pathogenesis of endometriosis remains unclear, it is generally accepted that its basic feature is the existence of ectopic endometrium. The chronic inflammatory reaction induced by ectopic endometrial lesions is the central process leading to endometriosis-related pain[4,5]. The resulting painful symptoms, such as dysmenorrhea, dyspareunia, and chronic pelvic pain, have a pronounced negative effect on quality of life and are the primary reason for which patients seek treatment[6,7]. Recent studies have emphasized the adverse effect of endometriosis symptoms on sexual function, work productivity, and psychological aspects of life[8]. The physical and mental suffering of patients is possibly due to the absence of sufficiently effective and targeted therapeutic methods.

The management of endometriosis has continuously improved, and a patient's choice of treatment methods, such as medication, surgery, or assisted reproductive technology, usually depends on her age and fertility requirements[4]. Medication is the first-line therapeutic option for women who do not wish to conceive in the immediate future, considering that surgery has a high rate of recurrence and risk of complications and can reduce the ovarian reserve[1]. There are also many widely available pharmacological treatments for endometriosis, such as painkillers, non-steroidal anti-inflammatories (NSAIDs), combined oral contraceptives (COCs), progestins, and gonadotrophin-releasing hormone analogues (GnRH-a)[9,10]. Although each type of drug is widely used worldwide, they also have clear disadvantages. Additionally, no drug has been proven markedly superior to others.

Dienogest, a 19-nortestosterone and progesterone derivative, is a fourth-generation synthetic oral progestin that was designed to treat endometriosis. It binds highly selectively to progesterone receptors, thereby exerting potent progestogenic effects

with little androgenic, mineralocorticoid, or glucocorticoid activity[11]. Crucially, dienogest suppresses the growth of endometrioid tissue and also exerts anti-inflammatory, antiproliferative, and antiangiogenic effects[12-14]. It has also shown good effectiveness and a favorable safety profile in long-term clinical application and follow-up studies. However, it remains uncertain whether dienogest is superior to other drugs.

The aim of this systematic review and Meta-analysis was to evaluate the efficacy and safety of dienogest for the treatment of endometriosis in women of reproductive age. We integrated and Meta-analyzed data from high-quality randomized controlled trials (RCTs) to assess differences between the treatment effects of dienogest and those of other drugs. This revealed strong evidence that dienogest should be the drug of first choice for treating endometriosis.

MATERIALS AND METHODS

Search strategy

To identify all relevant literature, a systematic search of the Cochrane Library, EMBASE, PubMed, and the Web of Science was conducted on January 21, 2020, using combinations of the following search terms: (endometriosis OR adenomyosis OR EMT OR EM OR uterine adenomyosis OR endometrioma) AND dienogest AND (placebo OR blank control OR drug). The search process and determination of eligibility were conducted independently by two investigators (Wang XF and Fu XL). Disagreements about eligibility or search criteria were mitigated through discussion with a third reviewer (Lin SC).

Study selection

We only included trials in which women had signs and symptoms of endometriosis or adenomyosis. Trials that met all of the following criteria were included: (1) Prospective RCTs; (2) English language studies; (3) Patients in the experimental group were treated with dienogest, whereas those in the control group received other medications, such as GnRH-a, placebo, COCs, or NSAIDs; and (4) The primary outcome was an improvement in endometriosis-associated pain, and other outcomes included adverse effects and changes in clinical laboratory parameters.

Meta-analyses, reviews, case reports, conference abstracts, cohort studies, retrospective studies, and trials without available data were excluded. Studies involving adolescent or menopausal women were also excluded.

Data extraction

Two authors (Wang XF and Fu XL) independently extracted the data from the studies that met all of the inclusion criteria and none of the exclusion criteria. The data extracted were the first author's name, the publication year, the study design, the interventions, the number of participants, and the inclusion criteria.

The primary efficacy outcome was a change in the visual analogue scale (VAS) scores of endometriosis-related pain before and after the trial, and the secondary efficacy outcome was a change in the number of analgesics administered. The outcomes used to evaluate safety were changes in bone mineral density (BMD) and estrogen concentration, the incidence of hot flushes and headaches, and the occurrence of other adverse events.

Study quality

To assess the quality of each included study, the Cochrane risk-of-bias assessment tool was used. Each type of risk was graded as low, high, or unclear for the included RCTs. Seven criteria related to the risk of bias were assessed in each study: (1) Random sequence generation; (2) allocation concealment; (3) blinding of participants and personnel; (4) blinding of outcome assessment; (5) incomplete outcome data; (6) selective reporting; and (7) other bias. Two authors (Wang XF and Fu X) independently assessed the risk of bias for each included study. Disagreements were resolved by discussion with a third reviewer (Lin SC).

Statistical analysis

Review Manager (RevMan) 5.3 software was used to conduct the Meta-analysis. Continuous data and dichotomous data are reported as the mean ± standard deviation (SDs) and the number of outcome events/total number, respectively. We also calcu-

lated the mean difference (MD) and 95% confidence intervals (CIs) of continuous data. Risk ratios (RRs) and 95%CIs were calculated for dichotomous data. *Q* tests and I^2 statistics were applied to evaluate the heterogeneity in outcomes between studies. If there was no heterogeneity or $I^2 < 50\%$, a fixed-effect model was used to analyze the results. If $I^2 \geq 50\%$, indicating the existence of high heterogeneity between studies, a random-effects model was used. Subgroup analysis was carried out according to the type of intervention. We also conducted a sensitivity analysis to find the source of heterogeneity. A funnel plot was used to assess publication bias. In all analyses, a *P*-value < 0.05 was considered statistically significant.

RESULTS

Study selection and characteristics

The search strategy identified 1028 trials. Initially, 385 duplicates were excluded, and a further 629 trials were excluded based on the title and/or abstract. The remaining 14 trials were further assessed by reading the full text. Four trials were excluded because randomization was not adopted in the grouping process[15-18]. One dose-ranging study was excluded because it did not compare dienogest with other treatments[19]. One trial's detailed data were not provided, and it was also excluded as the author did not respond to a request for data[20]. One trial investigating the benefits of dienogest treatment before *in vitro* fertilization and embryo transfer was excluded, as it did not measure the specific outcomes analyzed in this meta-analysis[21]. Finally, seven RCTs with a high level of evidence were eligible for the analysis[22-28]. The procedure for study selection is presented in Figure 1.

Table 1 presents the characteristics of the studies included in this meta-analysis. The seven prospective RCTs included a total of 1493 women from ten countries: Germany, Italy, Ukraine, Austria, Spain, Poland, Portugal, Japan, China, and Egypt. Two milligrams of dienogest per day was the dosage in all studies. Efficacy was assessed in terms of changes in VAS scores for endometriosis-related pain and changes in the intake of supportive analgesic medication (SAM). Safety was assessed in terms of changes in BMD and serum estradiol (E2) concentrations, and the incidence of headaches and hot flushes. The results of quality assessment are shown in Figure 2. All eligible trials indicated a low risk of bias.

VAS score for endometriosis-related pain

All seven studies assessed the efficacy of dienogest in comparison with that of other drugs (placebo or GnRH-a) in terms of changes in VAS scores at the end of the treatment period. However, the data of one study were unclearly presented[26]. Our meta-analysis revealed a statistically significant advantage of dienogest, compared with other drugs, in terms of the relief of endometriosis- or adenomyosis-associated pain (MD = -17, 95%CI: -30.19 to -3.80). Considering the rather high heterogeneity between the studies [an I^2 of 97% ($P < 0.00001$)], we conducted a subgroup analysis of the different categories of alternative drugs.

Dienogest was found to be significantly superior to GnRH-a for pain relief (MD = -2.41, 95%CI: -3.58 to -1.24) with a very low heterogeneity ($I^2 = 0.0\%$, $P = 0.57$). Dienogest was also significantly superior to placebo with respect to pain relief [MD = -32.93, 95%CI: -44.63 to -21.23], but with a high heterogeneity ($I^2 = 84\%$, $P = 0.002$) (Figure 3A). Subgroup analysis reduced the heterogeneity within each group but did not eliminate it. Therefore, we performed a sensitivity analysis by excluding each study, and then analyzing the effect of this exclusion. This revealed that the RCT performed by Lang *et al*[25] was the probable source of heterogeneity, as its elimination decreased I^2 from 84% to 0.0%. The combined results of the other two articles also indicated that dienogest was superior to lacebo for pain relief (MD = -38.83, 95%CI: -45.17 to -32.49). Visual inspection of the funnel plot of the changes in VAS scores reveals its asymmetry, which suggests that there was some degree of publication bias and that more studies are needed to validate the results (Supplementary Figure 1).

SAM intake

Three studies that compared dienogest with placebo reported the change in the level of SAM intake, and the combined results indicated a significantly larger reduction of SAM intake in the dienogest group than the placebo group (MD = -0.39, 95%CI: -0.75 to -0.03), with an I^2 of 72% ($P = 0.03$) (Figure 3B). Subgroup analyses of two studies (Osuga *et al*[27] and Lang *et al*[25]) demonstrated a high heterogeneity, *i.e.*, a minimum

Table 1 Characteristics of the studies included in the Meta-analysis

Ref.	Study design	Country	Blind	Duration	Intervention	Sample size	Efficacy assessments	Safety assessments
Harada *et al* [22], 2009	RCT	Japan	Double-blind	24 wk	2 mg/d DNG *vs* 900 μg/d buserelin	137/134	VAS	BMD change Hot flashes Headache
Strowitzki *et al*[23], 2010	RCT	Germany Austria Spain Poland Italy Portugal	Open-label	24 wk	2 mg/d DNG *vs* 3.75 mg/4wk leuprolide	124/128	VAS	Hot flashes Headache
Abdou *et al* [24], 2018	RCT	Egypt	Open-label	12 wk	2 mg/d DNG *vs* 3.75 mg/4 wk leuprolide	130/131	VAS	Hot flashes Headache
Lang *et al*[25], 2018	RCT	China	Double-blind	24 wk	2 mg/d DNG *vs* placebo	130/132	VAS SAM intake	BMD change E2
Strowitzki *et al*[26], 2010	RCT	Germany Italy Ukraine	Double-blind	12 wk	2 mg/d DNG *vs* placebo	102/96	SAM intake	BMD change E2 Headache
Osuga *et al* [27], 2017	RCT	Japan	Double-blind	16 wk	2 mg/d DNG *vs* placebo	34/33	VAS SAM intake	E2 Hot flashes
Harada *et al* [28], 2017	RCT	Japan	DNG: Un-blind Placebo: Blind	24 wk	2 mg/d DNG *vs* placebo	53/129	VAS	

EM: Endometriosis; AD: Adenomyosis; SAM: Supportive analgesic medication; BMD: Bone mineral density; E2: Serum estradiol.

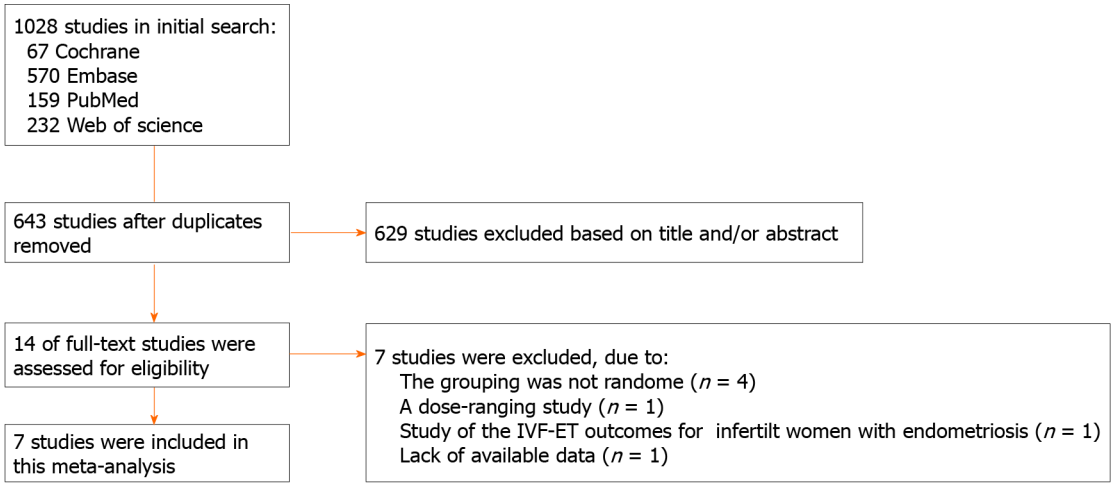


Figure 1 Flowchart of study selection process.

I^2 of 52%. The sensitivity analysis subsequent to removal of either of those studies indicated that the statistical difference between dienogest and placebo was not significant (P = 0.08 and 0.24 for Osuga *et al*[27] and Lang *et al*[25], respectively). Thus, additional studies are required to validate this result.

Change in BMD

Three studies investigated the effect of dienogest on BMD, and we found that there was a statistically significant difference between dienogest and the other drugs with respect to changes in BMD. Two studies compared dienogest with GnRH-a, whereas one study compared dienogest with placebo. Therefore, a subgroup analysis was conducted based on the type of drug used as the control. A greater reduction in BMD was observed in the GnRH-a group than in the dienogest group (MD = 2.77, 95%CI: 0.16 to 5.37), but there was a significant heterogeneity between these two trials (P = 0.02, I^2 = 80%). Furthermore, a smaller reduction in BMD was observed in the placebo group than in the dienogest group (MD = -0.71, 95%CI: -0.72 to -0.70) (Figure 4A).

Change serum E2 concentrations

Serum E2 concentrations were measured in three studies in which dienogest was

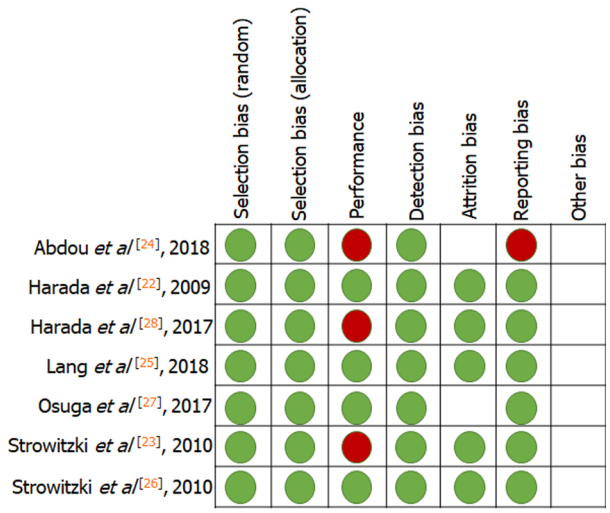


Figure 2 Risk of bias summary of randomized controlled trials.

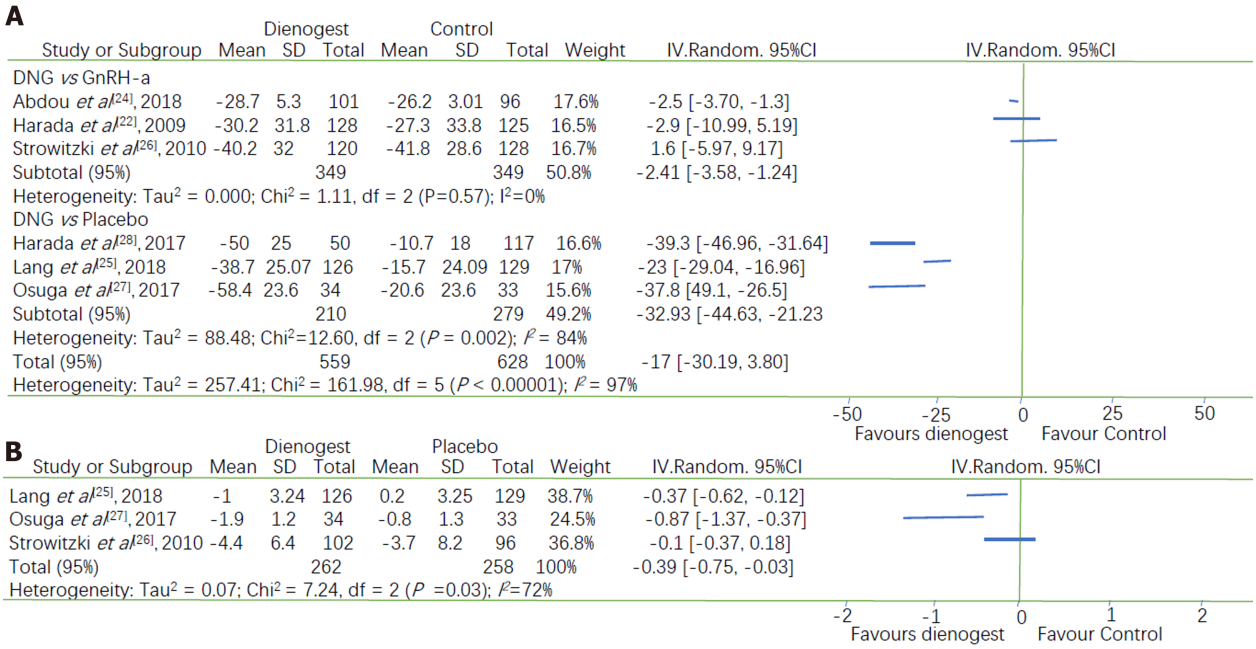


Figure 3 Change in visual analogue scale scores and supportive analgesic medication. A: Forest plot showing dienogest *vs* other intervention (GnRH-a and placebo) in the outcomes of changes in endometriosis related pain measured on visual analogue scale; B: Forest plot showing dienogest *vs* placebo in the outcome of supportive analgesic medication intake changes at the end of treatment. CI: Confidence interval.

compared with placebo. The E2 concentrations were lower in the dienogest groups than in the placebo groups. A Meta-analysis of the three studies showed that the decrease in the E2 concentration was -44.47 pg/mL [95%CI: -62.24 to -24.69]. The pooled measures revealed an I^2 value of 0.0% (P = 0.78), indicating a homogeneity between studies (Figure 4B).

Headache

Headaches were one of the most common drug-related adverse effects reported during treatment. Four studies reported the incidence of headache after treatment: Three studies comparing dienogest with GnRH-a and one comparing dienogest with placebo. Their I^2 of 31% (P = 0.23) indicated that there was a high heterogeneity between these studies. Therefore, the studies were divided into two subgroups according to the type of drug used in the control group. Women in the dienogest group were less likely to experience headaches than those in the GnRH-a group (RR = 0.68, 95%CI: 0.52 to 0.91), with I^2 of 0.0% indicating that there was no heterogeneity between the studies in this group. The only RCT that compared dienogest with a

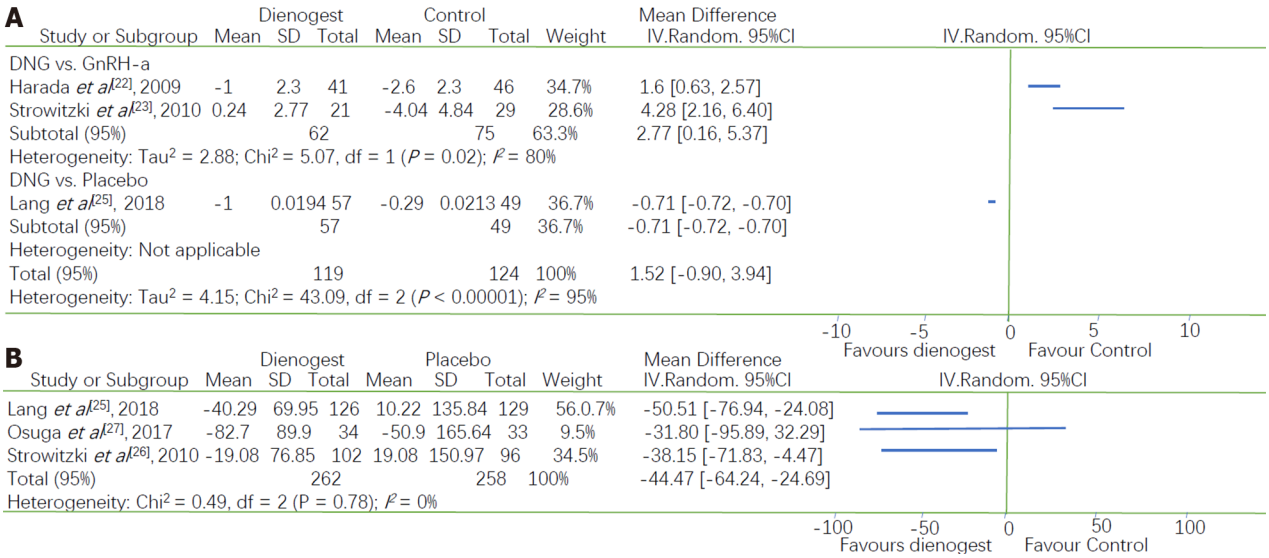


Figure 4 Changes in bone mineral density and serum estradiol. A: Forest plot showing dienogest *vs* other intervention (GnRH-a and placebo) in the outcomes of percent change in bone mineral density; B: Forest plot showing dienogest *vs* placebo in the change of serum estradiol at the end of treatment. CI: Confidence interval.

placebo did not show a significant difference in the incidence of headaches (RR = 2.07, 95%CI: 0.75 to 5.74, P = 0.16) (Figure 5A).

Hot flushes

Three studies comparing dienogest with GnRH-a and one study comparing dienogest with placebo reported the incidence of hot flushes. Due to the high heterogeneity between these studies (I^2 = 81%), we performed a subgroup analysis based on the type of drug used as the control. Only one study compared dienogest with placebo, reporting that the difference in the incidence of hot flushes was not statistically significant (RR = 4.86, 95%CI: 0.24 to 97.51).

The subgroup analysis showed that the incidence of hot flushes was greater in women treated with GnRH-a than in women treated with dienogest (RR = 0.43, 95%CI: 0.18 to 1.02; I^2 = 86%, P = 0.0008) (Figure 5B). After excluding the study by Harada *et al* [22] from the sensitivity analysis, the I^2 decreased from 86% to 39%. However, the combined results of the two remaining studies indicated that the difference between dienogest and GnRH-a with respect to the incidence of hot flushes was not statistically significant (P = 0.06). Given these conflicting results, more data are required to clarify the differences between the incidence of this adverse effect resulting from dienogest or GnRH-a treatment.

DISCUSSION

The chronic nature of endometriosis means that lifelong management must be the focus of clinical decision making, for which patients urgently need safer and more effective drugs. To comprehensively evaluate the efficacy and safety of dienogest in the treatment of endometriosis, we performed a Meta-analysis of seven RCTs that included a total of 1493 patients. The results showed that the pain-relieving ability of dienogest was superior to that of placebo and GnRH-a. Dienogest also exhibited significant safety advantages over GnRH-a, due to its low incidence of adverse effects. Taken together, these findings indicate that dienogest should be the first-line treatment for endometriosis.

The superior pain-relieving ability of dienogest may be attributable to the greater decrease in serum E2 concentrations observed in dienogest-treated patients than in those who received other treatments. This is consistent with the fact that a randomized, dose-controlled study indicated that a daily 2-mg dose of dienogest moderately suppresses E2 production and reliably inhibits ovulation[29], and that a moderate decrease in estrogen concentration effectively suppresses the growth of endometrial tissue and does not result in hypoestrogenic adverse effects, in accordance with the threshold theory proposed by Barbieri[30]. The patients' dependence on

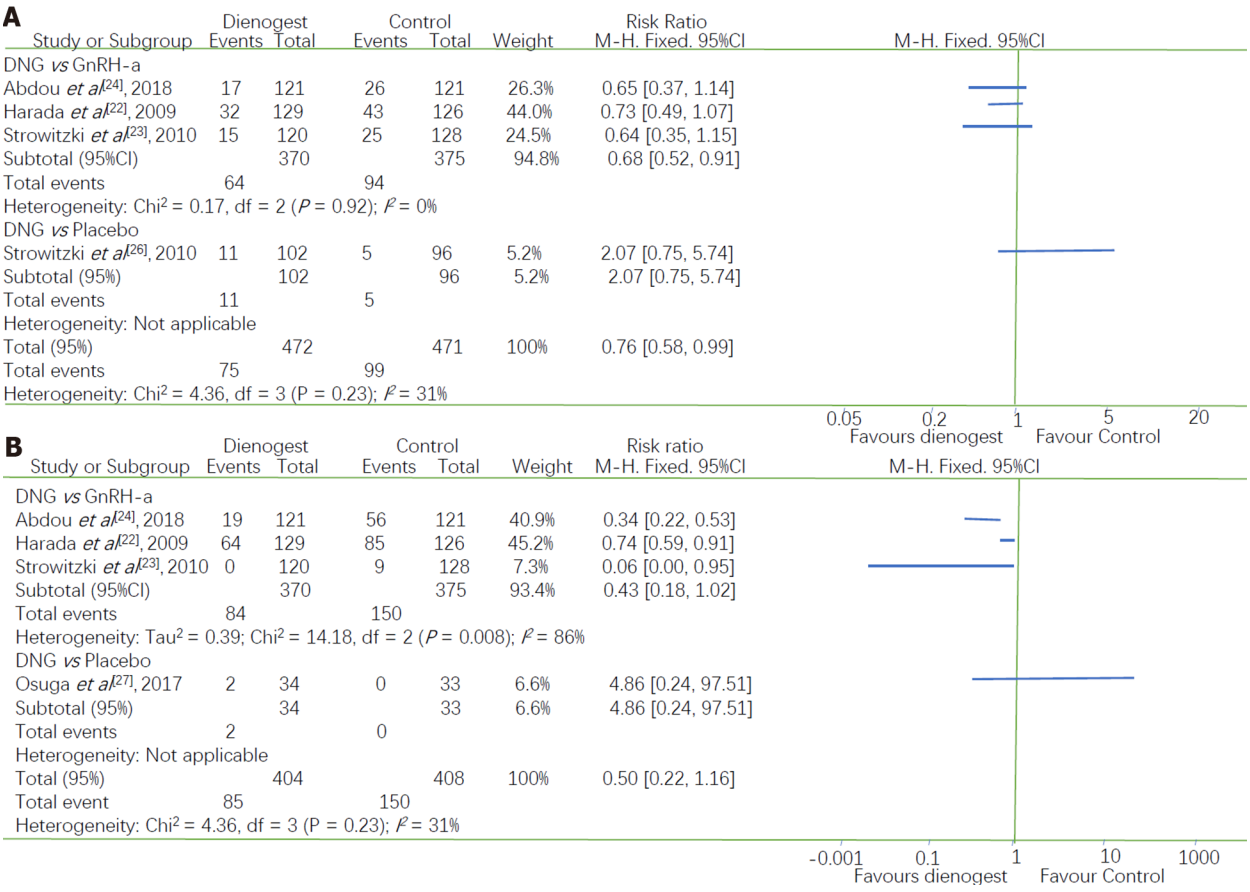


Figure 5 Incidence of headache and hot flushes. A: Forest plot showing dienogest *vs* other intervention (GnRH-a and placebo) in the incidence of headache; B: Forest plot showing dienogest *vs* other intervention (GnRH-a and placebo) in the incidence of hot flushes.

supportive analgesics also decreased during treatment with dienogest, providing indirect evidence for the drug's pain-relieving effect. In addition, in the safety analysis, no difference was observed in the incidence of headaches and hot flushes between the two groups. An extended study investigating the effects of dienogest treatment over 53 wk found that it induced a sustained reduction in endometriosis-associated pelvic pain, with low rates of treatment-related adverse events[31].

The superiority of dienogest over GnRH-a for relieving endometriosis-related pain was also supported by a -2.41 mm difference in VAS pain scores. In concordance with our findings, another meta-analysis concluded that dienogest was superior to GnRH-a in this regard, with a -2.17 mm difference in VAS pain scores[32]. Gerlinger *et al*[33] suggested a non-inferiority margin of 10 mm for endometriosis related pian measured using a VAS. Therefore, although the -2.41 mm difference in the VAS pain score is statistically significant, it is less than the suggested minimal clinically significant difference of 10 mm, and thus does not prove that dienogest is superior to GnRH-a in relieving endometriosis-related pain. However, the two drugs appear equivalent in terms of their pain-relieving ability, and more RCTs are required to confirm whether they clinically differ in this ability.

The safety of drugs used for the long-term management of endometriosis must not be overlooked. Dienogest showed significant safety benefits over GnRH-a, as the latter was more likely to lead to decreased BMD and an increased incidence of headaches and hot flushes. GnRH-a are an effective therapy for endometriosis, but they are also associated with hypoestrogenism and decreased BMD if taken for more than 6 mo. Patients are prescribed an add-back therapy to prevent this adverse event[34]. In contrast, dienogest can be taken for a long term with fewer adverse effects. A pooled analysis from four European RCTs confirmed the favorable safety and tolerability of dienogest in both short- and long-term use[35]. Thus, dienogest appears a better option for patients who require chronic treatment.

Research on the mechanism by which dienogest relieves the pelvic pain of endometriosis remains exploratory. Some researchers have found that dienogest can inhibit the production of tumor necrosis factor alpha and interleukin 1 beta by agonizing progesterone receptors, and ultimately inhibit the production of nerve growth factor, which

has been shown to be an important factor in the pelvic pain of endometriosis[36-39]. It has also been suggested that the high efficacy of dienogest in relieving endometriosis pain is related to the expression of nuclear factor kappa-B (NF-κB) and Bcl-2, because dienogest can increase the activity of NF-κB and thus increase the apoptosis of endometrial mesenchymal cells[13,40]. Accordingly, a fuller understanding of the mechanism of action dienogest is required, as this may optimize its clinical applicability.

The main strength of this meta-analysis is that we only included RCTs with high levels of evidence, which included a total of 1493 patients, most of whom were clearly diagnosed by histology or laparoscopy. Second, our analysis of the safety and effectiveness of dienogest for the treatment of endometriosis was comprehensive and systematic. The results of this meta-analysis demonstrate how the clinical use of medications for endometriosis could be standardized and enhance patients' ability to select the best treatment.

However, there are some limitations of our study. First, the included studies differed in the category of disease, the ethnicity of participants, the types of GnRH-a used for treatment, the administration route, and the duration of treatment, which may account for the heterogeneity between studies that we observed. For example, in analyzing the changes in BMD between the two studies, Gerlinger *et al*[32] attributed the heterogeneity observed between these studies to ethnicity. Moreover, estrogen plays a crucial role in the growth and maintenance of the skeleton, and Asian women have higher blood concentrations of estrogens than Caucasian women[41]. In addition, we conducted subgroup and sensitivity analyses to determine the sources of heterogeneity. Second, although the most frequent drug-related effect of dienogest in all included studies was irregular uterine bleeding (spotting and breakthrough bleeding), our safety assessments did not analyze this side effect. Although patients used daily diaries to record bleeding patterns in four of the included trials, these data cannot be used to accurately estimate the severity of bleeding. As such, an approach that can quantitatively evaluate irregular genital bleeding is needed to investigate this adverse effect. Third, our meta-analysis only included a small number of studies, and none compared dienogest with other therapies, such as compound COCs or levonorgestrel intrauterine devices, which have been proven effective in the treatment of endometriosis and adenomyosis. Finally, more studies are required to evaluate the pharmacoeconomics of drugs that are commonly used to treat endometriosis.

CONCLUSION

This Meta-analysis systematically evaluated the efficacy and safety of dienogest for the treatment of endometriosis. The results showed that dienogest is superior to placebo and GnRH-a in terms of pain relief and is better tolerated than GnRH-a. This demonstrates that dienogest may be the best medication for endometriosis patients seeking pain relief. Further high-quality RCTs are warranted to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Endometriosis is one of the common gynecological diseases in reproductive women and the concomitant pelvic pain has substantial negative effects on patients' quality of life. In recent years, significant advances have been made in the treatment of endometriosis, and drugs remain the primary treatment option for women of childbearing age. However, there is no agreement on which drugs are most effective and tolerated for the treatment of endometriosis.

Research motivation

Several well-designed randomized controlled trials (RCTs) have demonstrated that dienogest is effective in relieving endometriosis-related pain and has a tolerable adverse-effect profile. However, these RCTs have not unambiguously demonstrated whether dienogest is superior to other drugs.

Research objectives

This study was conducted to evaluate the effectiveness and safety of dienogest compared with other drugs for the treatment of endometriosis.

Research methods

This meta-analysis only included RCTs that compared dienogest with other drugs in the treatment of endometriosis. Review Manager (RevMan) 5.3 software was used to calculate mean difference (MD) values and risk ratios (RRs) with 95% confidence intervals (CIs).

Research results

This study included seven RCTs with 1493 participants and demonstrated that dienogest was more effective than placebo in alleviating endometriosis-related pain (MD = -32.93, 95%CI: -44.63 to -21.23), and led to a more significant decrease in plasma estradiol concentrations than placebo (MD = -44.7, 95%CI: -62.24 to -24.69). The combined results showed that dienogest was superior to GnRH-a in relieving pain (MD = -2.41, 95%CI: -3.58 to -1.24). Furthermore, adverse events were more frequent in patients in the GnRH-a group, including the loss of BMD (MD = 2.77, 95%CI: 0.16 to 5.37), headaches (RR = 0.68, 95%CI: 0.52 to 0.91), and hot flushes (RR = 0.43, 95%CI: 0.18 to 1.02).

Research conclusions

Dienogest is an effective and tolerable therapeutic for the treatment of endometriosis-related pain in women of reproductive age.

Research perspectives

The results of this meta-analysis provide insights on how the clinical use of medications for endometriosis could be standardized and should improve the choice of medications for patients.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 August 28; 9(4): 389-404

DOI: 10.13105/wjma.v9.i4.389

ISSN 2308-3840 (online)

SCIENTOMETRICS

Trends in iron deficiency anemia research 2001-2020: A bibliometric analysis

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Author contributions: The manuscript was conceived, designed, and written by Frater JL.

Conflict-of-interest statement: The author declares no conflict-of-interest related to this article.

PRISMA 2009 Checklist statement: The author has read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Manuscript source: Invited manuscript

Specialty type: Medicine, research

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Abstract

BACKGROUND

Iron deficiency anemia has a worldwide impact on individual health and national and international economies, with an estimated one-third of the world population being iron deficient.

AIM

To evaluate the iron deficiency literature published between 2001-2020 with an emphasis on: (1) Identification of collaborative research networks most active in this field; (2) Research topics of greatest importance; and (3) Analysis of the most-cited papers published between 2001-2020 and the most cited papers in 5-year intervals during this period to assess for emerging trends in research in this area.

METHODS

A search of Clarivate Analytics World of Science Core Collection was performed for the topic "iron deficiency anemia", limited to document type (article or review), language (English), and time span (2001-2020). The following data were extracted from these articles: Year of publication, journal, study design, country of first author, and number of citations. The metadata derived from the search were used to identify publication trends in iron deficiency anemia research and their distribution in countries/regions and institutions. Network visualization by VOSviewer (Leiden University) was performed to identify international collaborative groups and research hotspots.

RESULTS

The search identified 4828 publications. Three international collaborative networks were identified: United States, Canada, and India; Turkey, China, and Japan; and England and other European countries. Five research areas were hotspots: Epidemiologic aspects of iron deficiency anemia, biochemical aspects of iron deficiency anemia, clinical evaluation of causes of iron deficiency anemia, causes of iron deficiency anemia, and bioavailability of dietary iron. Subset analysis of the top-10 overall cited papers, and the top-10 cited papers for each 5-

and experimental

Country/Territory of origin: United States

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E

Received: January 31, 2021
Peer-review started: January 31, 2021
First decision: May 6, 2021
Revised: May 17, 2021
Accepted: July 12, 2021
Article in press: July 12, 2021
Published online: August 28, 2021

P-Reviewer: Ho YS, Rodrigo L
S-Editor: Fan JR
L-Editor: A
P-Editor: Li JH

year increment beginning in 2001 showed that the largest number of highly cited papers were from the field of epidemiology, the smallest number from the field of bioavailability of dietary iron.

CONCLUSION
The literature on iron deficiency anemia has a high citation rate compared to studies of other topics using similar methodology and is heavily biased toward studies from the United States and epidemiologic studies.

Key Words: Iron deficiency anemia; Bibliometrics; VOSviewer; Trends

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Core Tip: Iron deficiency is a common micronutrient deficiency with a worldwide impact. This bibliometric analysis was performed to analyze the literature on iron deficiency published between 2001-2020. Three international collaborative networks based in North America/India, Europe, and Asia were identified. There are 5 areas of greatest focus: Epidemiologic aspects of iron deficiency anemia, biochemical aspects of iron deficiency anemia, clinical evaluation of iron deficiency anemia, causes of iron deficiency anemia, and bioavailability of dietary iron. Evaluation of the papers published during this period identified epidemiology as the most cited area, and bioavailability of iron as the least cited.

Citation: Frater JL. Trends in iron deficiency anemia research 2001-2020: A bibliometric analysis. *World J Meta-Anal* 2021; 9(4): 389-404
URL: https://www.wjgnet.com/2308-3840/full/v9/i4/389.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i4.389

INTRODUCTION

Iron deficiency and iron deficiency anemia

Iron deficiency and iron deficiency anemia are health conditions with a worldwide effect on individual health and an impact on national and international economies. An estimated one-third of the world population is affected by iron deficiency, and deficiency of this micronutrient has effects on multiple systems of the body, including the central nervous system and reticuloendothelial systems[1,2]. Health consequences of iron deficiency are widespread and include impaired intellectual function and decreased immunity[3,4].

Anemia is a common consequence of iron deficiency. The degree of severity of anemia is variable, and it is microcytic and hypochromic as demonstrated by routine laboratory testing, which identifies a moderate to severe decrease in red blood cell count, hemoglobin, and hematocrit, accompanied by a moderately decreased mean corpuscular volume and moderate to marked elevation of red blood cell distribution width[1].

The bibliometric method as a research tool

Bibliometric methods are useful to evaluate trends in research activities over time[5]. Bibliometrics take advantage of literature databasing technology, including literature metrology, and is an increasingly important method of providing insight into research in specific fields. This format has been used to evaluate the impact of articles in many areas of study. It is valuable to identify the most cited studies that have influenced the evolution of a given scientific field[6].

Rationale

In this study, the research trends in the field of iron deficiency anemia in the past 20 years were considered. The aims of this study were to: (1) Identify and analyze scientific publications in this field; and (2) Compare the contribution of this research in different countries and institutions. Bibliometric analysis was performed using the functions of the Web of Science Core Collection and further analysis of the metadata

was performed using VOSviewer software (Leiden University). Using the features of VOSviewer, a network visualization of international collaborations and a keyword-based visualization of research fields was performed. This study provides a refined understanding of global trends in iron deficiency anemia research.

MATERIALS AND METHODS

This bibliometric analysis was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) principles (see PRISMA 2009 checklist statement for PRISMA checklist) according to a methodology similar to that described in earlier studies[6-10], which is detailed below.

Data collection and bibliometric analysis

All articles were searched using the Clarivate Analytics World of Science Core Collection (WOSCC) on August 30, 2020. The study used publicly available data, and thus ethical approval was not required. The search criteria were topic ("iron deficiency anemia"), limited to document type (article or review), language (English), and time span (2001-2020). A topic search includes a search of the following fields: The title of the article or review; the abstract, the keywords; and the keywords plus©, which is a proprietary algorithm using expanded terms from an article's cited references or bibliography. The following data were extracted from these articles: Year of publication, journal, study design, country of first author, and number of citations. The metadata derived from the search were used to identify publication trends in iron deficiency anemia research and their distribution in countries/regions and institutions. This protocol has not been registered.

VOSviewer bibliometric software (Van Eck and Waltman, Leiden University, Leiden, The Netherlands) was used to perform data mining, mapping, and clustering of the retrieved articles[11]. Keywords and countries were labeled with colored circles, the size of which correlated with the occurrence of the keyword or countries in the title and abstract.

Subset analysis of abstracts/full-text

Because of the large number of search results, the title, abstract and full text of the top 200 cited articles were reviewed for appropriateness to the topic. The entire list of retrieved articles was analyzed, and an additional subset analysis of the articles grouped by publication in 5-year increments (2001-2005; 2006-2010; 2011-2015; 2016-2020) was performed. The top 25 keywords identified in the title and abstract of each publication in each time interval was compiled[12]. In addition, the top 10 articles published in each time interval was identified, along with their number of citations.

RESULTS

The search returned 4828 references. Review of the titles, abstracts, and full texts of the top 200 cited papers in this group was performed to assess the quality of the search, and all papers in this group were appropriate to the topic of iron deficiency anemia.

These publications had an h-index of 137 with an average of 25.42 citations *per* item. The number of papers published *per* year in this study has varied from 124 to 402. The year with the largest number of papers published in this study was 2019. The rate of publication of papers in this study has varied from 2.568% to 8.326% (Figure 1).

Of 4163 papers were classified as articles, 695 as reviews. 4569 papers (94.6% of the total) were published in English. Based on WOSCC metadata, the papers were published in 97 different research areas, of which the most common were nutrition and dietetics (n = 672, 13.919% of total), gastroenterology (n = 610, 12.635%), hematology (n = 570, 11.806%), pediatrics (n = 566, 11.723%), and general internal medicine (n = 522, 10.812%).

Country of publication

In total, publications were contributed by 157 countries, with the top ten publishing countries listed in Figure 2.

The United States has contributed the largest number of the papers. Other nations in the top 5 countries of publication were Turkey, China, Italy, and England. The authors in this study represented 4840 institutions. The institutions contributing the most

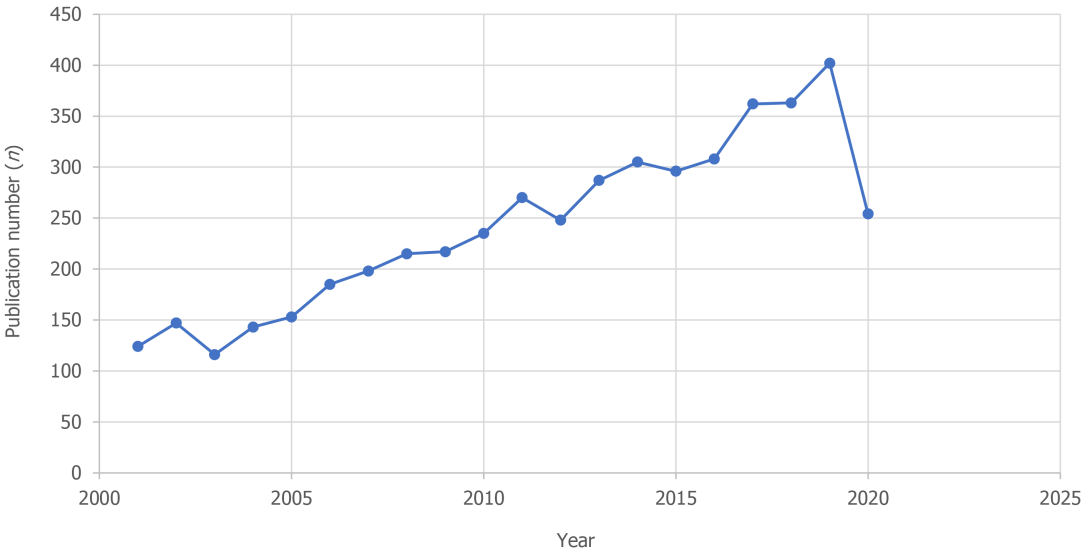


Figure 1 Number of publications (*n*) *per* year, 2001-2020.

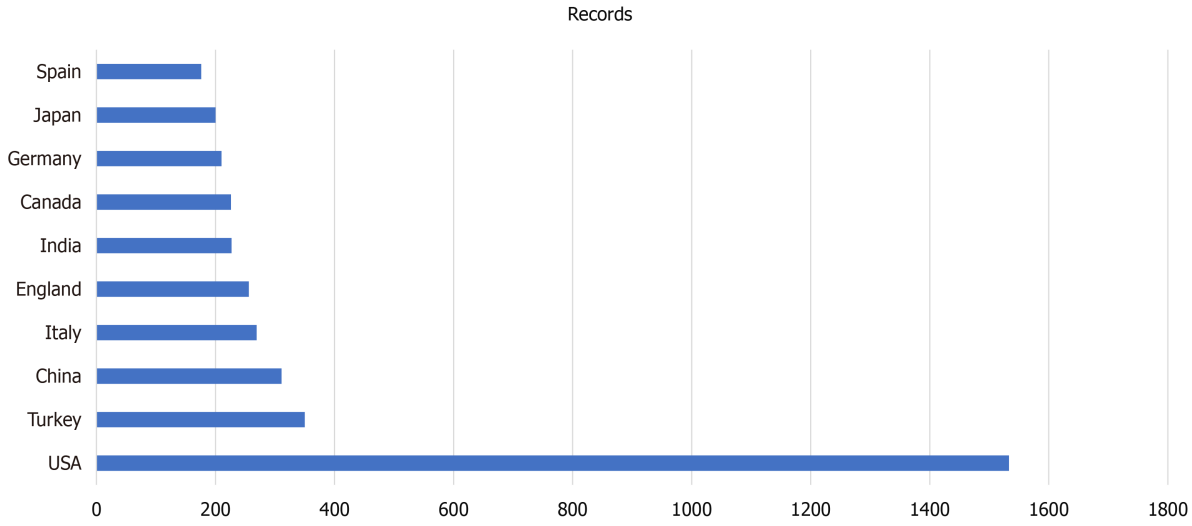


Figure 2 Number of records (*n*) *per* country for the top 10 countries contributing papers to this study.

papers to this study were the University of California system (n = 179 records) and Harvard University (n = 126 records). 2411 funding agencies were listed in these publications, of which the largest number of studies were funded by the United States Department of Health and Human Services (n = 448), the National Institutes of Health (United States, n = 431), and the National Natural Science Foundation of China (n = 119).

Collaborations

The collaboration network analysis is illustrated in Figure 3 and includes countries contributing at least 10 papers. Using this criterion, 64 countries are included in the analysis. There are 3 nodes identified using international collaboration data. The largest, illustrated in red, includes the United States, Canada, and India as the largest contributors. The second, illustrated in blue, includes Turkey, China, and Japan as the most prominent contributing members. The third, illustrated in green, includes England and many European countries.

Journals

The papers in this study were published by 1365 journals. 659 journals published ≥ 1 paper; the remaining journals published 1 paper apiece. The top 15 journals, with the number of articles published and the journal's impact factor (IF), drawn from the 2020

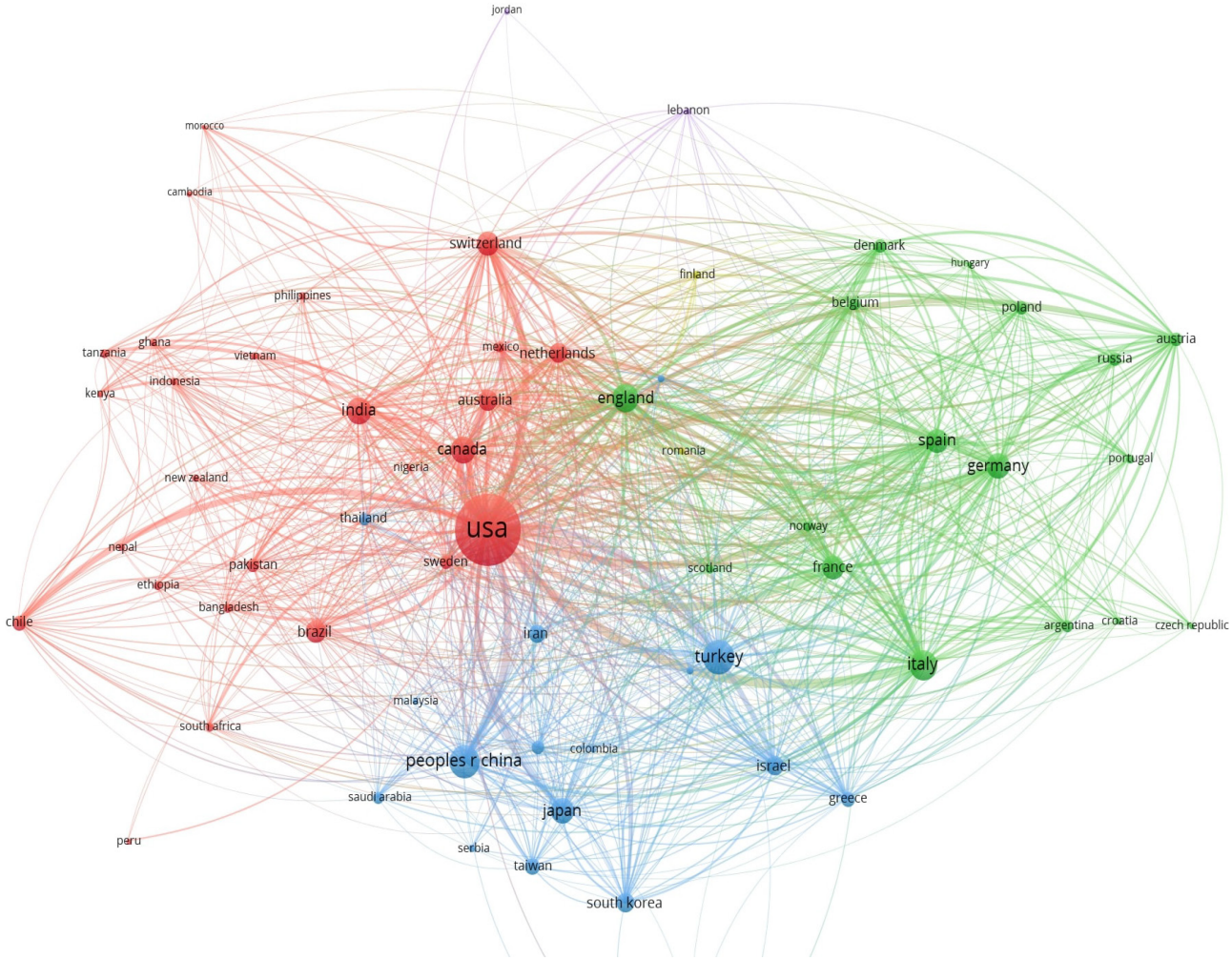


Figure 3 Collaboration network analysis of the 64 countries contributing ≥ 10 papers.

Journal Citation Reports of Clarivate Analytics) are shown in Table 1. The IF for the journals in this group ranged from 17.543 (*Blood*) to 1.016 (*Journal of Pediatric Hematology Oncology*). The largest number of papers were published by *Journal of Nutrition* (n = 107), *PLOS One* (n = 81), and *World Journal of Gastroenterology* (n = 76).

Co-occurrence of keywords

Overall, 123 terms appeared 50 times or more in the titles or abstracts of the papers in this study (Figure 4). For example, "iron deficiency anemia" appeared 1533 times, "anemia" appeared 1252 times, "children" appeared 726 times, "iron deficiency" appeared 627 times, and "prevalence" appeared 608 times. Based on the VOSviewer keyword mapping, the terms or phrases associated with iron deficiency anemia were divided into 5 clusters, represented by 5 colors (red, green, blue, yellow, and purple). From the results of co-occurrences, current iron deficiency anemia research was shown to be mainly focused on 5 major areas. These are: (1) Epidemiologic aspects of iron deficiency anemia (red); (2) Bioavailability of dietary iron (purple); (3) Clinical evaluation of causes of iron deficiency anemia (blue); (4) Causes of iron deficiency anemia (yellow); and (5) Biochemical aspects of iron deficiency anemia (green). These 5 topics may thus be regarded as the research hotspots in the field of iron deficiency anemia between 2001-2020.

A subset analysis of the top 25 author-selected keywords of each 5-year interval between 2001-2020 and coded to the 5 areas presented in Figure 4 is presented in Table 2[12]. The distribution of keywords was relatively unchanged over the years. The largest number of keywords mapped to the epidemiologic aspects of iron deficiency anemia (red) cluster (11-12 keywords in each interval), with smaller numbers of keywords mapping to the other clusters.

Table 1 Top 15 journals ranked by number of papers published, and corresponding impact factors

Journal	Records	Impact factor
Journal of nutrition	107	4.281
PLoS One	81	2.740
World Journal of Gastroenterology	76	3.665
American Journal of Clinical Nutrition	75	6.766
Nutrients	67	4.546
Pediatrics	50	5.359
Food	48	1.485
Journal of Pediatric Hematology Oncology	48	1.016
Helicobacter	44	4.000
American	39	6.973
Blood	39	17.543
Digestive Diseases and Sciences	35	2.751
Pediatric Hematology and Oncology	34	1.232
Biological	33	2.639
Journal of Pediatric Gastroenterology and Nutrition	33	2.937

Top 10 cited papers

The top 10 cited papers published for the entire period 2001-2020, and the top 10 cited papers published in each 5-year interval are listed in Table 3. The total number of citations *per* paper for the top 10 cited papers published from 2001-2020 ranged from 752 to 4084.

DISCUSSION

This bibliometric analysis was performed to evaluate the research trends in the field of iron deficiency anemia between 2001-2020. The purpose of this study was: (1) To identify and analyze scientific publications in this field; and (2) To compare the contribution of this research in different countries and institutions. The main findings were (1) That the most common topic areas were nutrition and dietetics, gastroenterology, hematology, pediatrics, and general internal medicine; (2) United States-based researchers contributed to the vast majority of papers, although researchers from Turkey, China, Italy, and England also made significant contributions to the literature; (3) Keyword analysis revealed that 5 research areas have developed as current hotspots: Epidemiologic aspects of iron deficiency anemia, biochemical aspects of iron deficiency anemia, clinical evaluation of causes of iron deficiency anemia, causes of iron deficiency anemia, and bioavailability of dietary iron; and (4) Evaluation of the top keywords in 5 year intervals showed that the relative contributions of each research area to the total number of papers has remained static, with the largest contribution to the area of epidemiologic aspects of iron deficiency anemia. The citation rate of the top cited papers in this study is high compared to studies on other research areas using similar methodology[13].

Epidemiologic aspects of iron deficiency anemia

The topic of iron deficiency anemia has been the subject of numerous epidemiologic studies, including the Global Burden of Disease Study, a large-scale observational epidemiologic survey which was published as a 4-paper series[14-17]. This includes the top-cited paper identified in the current study[14]. Approximately a third of the world population is iron-deficient[18], though there are clear disparities in the distribution of iron deficiency. Darmon *et al*[19] established that iron deficiency anemia was more prevalent in individuals from low socioeconomic backgrounds, possibly due to diet quality[19]. Guralnik *et al*[20]'s 2005 paper identified that iron deficiency anemia contributes to a high percentage of cases of anemia in the elderly[20]. Although at-risk

Table 2 Top 25 keywords of each 5-year interval between 2001-2020 color coded to the clusters identified in Figure 4

2001	2006	2011	2016
Iron-deficiency anemia[3]	Iron-deficiency anemia[3]	Iron-deficiency anemia[3]	Iron-deficiency anemia[3]
Anemia[1]	Anemia[1]	Anemia[1]	Anemia[1]
Children[1]	Iron deficiency anemia[1]	Iron deficiency anemia[1]	Iron deficiency anemia[1]
Iron deficiency anemia[1]	Children[1]	Children[1]	Prevalence[1]
Iron deficiency[2]	Iron deficiency[2]	Iron deficiency[2]	Children[1]
Iron[5]	Prevalence[1]	Prevalence[1]	Iron deficiency[2]
Prevalence[1]	Diagnosis[3]	Iron[5]	Diagnosis[3]
Serum ferritin[5]	Iron[5]	Diagnosis[3]	Management[3]
Ferritin[5]	Supplementation[1]	Supplementation[1]	Iron[5]
Supplementation[1]	Infants[1]	Hepcidin[5]	Supplementation[1]
Diagnosis[3]	Serum ferritin[5]	Management[3]	Pregnancy[1]
Absorption[2]	Women[1]	Hemoglobin[1]	Hepcidin[5]
Deficiency[5]	Hemoglobin[1]	Pregnancy[1]	Hemoglobin[1]
Infants[1]	Absorption[2]	Ferritin[5]	Risk[3]
Erythropoietin[4]	Deficiency[5]	Intravenous iron[4]	Intravenous iron[4]
Pregnancy[1]	Hepcidin[5]	Women[1]	Women[1]
Women[1]	Therapy[4]	Deficiency anemia[1]	Ferritin[5]
Therapy[4]	Iron-deficiency[1]	Disease[3]	Oral iron[4]
Hemoglobin[1]	Growth[1]	Inflammation[5]	Health[1]
Eradication[3]	Helicobacter pylori[3]	Risk[3]	Disease[3]
Serum transferrin receptor[5]	Pregnancy[1]	Association[3]	Infants[1]
Helicobacter pylori[3]	Disease[3]	Metabolism[1]	Deficiency[5]
Iron-deficiency[1]	Risk[3]	Infants[1]	Iron-deficiency[1]
Deficiency anemia[1]	Celiac disease[3]	Serum ferritin[5]	Deficiency anemia[1]
Disease[3]	Expression[5]	Deficiency[5]	Ferric carboxymaltose[4]
Number of keywords			
Red			
11	12	11	12
Purple			
2	2	1	1
Blue			
5	5	6	5
Yellow			
2	1	1	3
Green			
5	5	6	4
New items in each quartile			
(None)	Serum ferritin[5]	Management[3]	Oral iron[4]
	Hepcidin[5]	Intravenous iron[4]	Health[1]
	Growth[1]	Inflammation[5]	Ferric carboxymaltose[4]
	Risk[3]	Association[3]	

	Celiac disease[3]	Metabolism[1]
	Expression[5]	Infants[1]

[1]Red.
[2]Purple.
[3]Blue.
[4]Yellow.
[5]Green.
Summary shows the number of keywords in each 5-year quartile by cluster and a list of the keywords appearing for the first time in the top-25 list for each quartile.

populations are particularly prevalent in the developing world[21], residents of industrialized national are also impacted, as noted by Dubé *et al*[22].

Additional highly cited papers in this study addressed the neurologic implications of iron deficiency and iron deficiency anemia. These include Grantham-McGregor *et al*[23]'s review of the long-term effects of iron deficiency on neurological functioning in children[23] and Beard and Connor's seminal paper on iron status and its correlation with neural functioning[24]. The work of Lozoff and colleagues, including their 2006 paper which is highlighted in the current study, detailed that the neurological defects in patients in iron-deficient children may extend into adulthood, even after correction of the underlying nutritional issue[25].

An important consequence of iron deficiency and iron deficiency anemia is decreased work output, which may have long-term impact on affected individuals and has an established societal effect. These features were described in highly-cited work by Haas and Brownlie[2].

Bioavailability of dietary iron

Although iron deficiency is a widespread international public health issue, few high-impact studies have been dedicated to the critical issue of iron bioavailability, as evidenced from the survey of top 10-cited papers. Two review papers have discussed features of iron bioavailability. The first, by Plum *et al*[26], examines the role of zinc in iron metabolism[26]. The second, by Xie *et al*[27], examines the physiologic role of polysaccharides derived from medicinal plants, some of which have been identified as having iron-chelating properties[27].

Biochemical aspects of iron deficiency anemia

Though none of the top-10 cited studies had iron metabolism as a main topic, 4 studies in the quartile analysis were focused on biochemical aspects of iron anemia. Three of these had a particular focus on hepcidin. Hepcidin, a protein produced by the liver and cleared by the kidneys, plays a central role in iron uptake by the duodenum and spleen. Its regulation was described in a highly cited paper identified in this study [28], which showed that hepcidin expression is upregulated in response to anemia and hypoxia and downregulated by increased hematopoiesis. An immunoassay for hepcidin was described by Ganz *et al*[29] and Girelli *et al*[30]. Laboratory testing for serum hepcidin levels can be used to distinguish iron deficiency anemia from anemia of chronic disease (inflammation)[1].

Another important step in iron metabolism highlighted by the current study is the action of ferroreductase, which acts on ferric (3+) iron-containing molecules in the cytoplasm of phagocytes to produce bioavailable ferrous (2+) iron[31,32].

Causes of iron deficiency anemia

Numerous health conditions have been implicated in the etiology of iron deficiency anemia. Among these are chronic inflammatory conditions, chronic gastritis (in particular gastritis secondary to *Helicobacter pylori*), chronic use/abuse of non-steroidal anti-inflammatory drugs (NSAID), and celiac disease. Iron deficiency may also follow gastrointestinal procedures such as bariatric surgery and capsule endoscopy[33]. The role of *Helicobacter pylori* gastritis and celiac disease were the subjects of 2 papers from the overall top 10-cited group[34-36]. In addition, Graham *et al*[37] identified the mucosal lesions in the gastrointestinal tracts of chronic NSAID users, many of whom developed iron deficiency anemia[37]. In patients with chronic kidney disease, decreased renal clearance of hepcidin may result in chronic kidney disease, as described by Levin and Rocco[38].

Table 3 The top 10 cited papers published for the entire period 2001-2020, and the top 10 cited papers published in each 5-year interval within the study period

Ref.	Title	Journal	Year	Volume	Start page	End page	Times cited	Topic
2001-2020								
Vos *et al*[14], 2012	Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010	*Lancet*	2012	380	2163	2196	4084	1
Malfertheiner *et al*[34], 2007	Current concepts in the management of Helicobacter pylori infection: the maastricht III consensus report	*Gut*	2007	56	772	781	1481	3
Bhutta *et al*[44], 2008	Maternal and Child Undernutrition 3 - What works? Interventions for maternal and child undernutrition and survival	*Lancet*	2008	371	417	440	1147	4
Darmon and Drewnowski[19], 2008	Does social class predict diet quality?	*American Journal of Clinical Nutrition*	2008	87	1107	1117	1061	1
Walker *et al*[45], 2007	Child development in developing countries 2 - Child development: risk factors for adverse outcomes in developing countries	*Lancet*	2007	369	145	157	1033	4
Chey and Wong[39], 2007	American college of gastroenterology guideline on the management of *Helicobacter pylori* infection	*American Journal of Gastroenterology*	2007	102	1808	1825	830	3
Guralnik *et al*[20], 2004	Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia	*Blood*	2004	104	2263	2268	793	1
Ludvigsson *et al*[40], 2013	The Oslo definitions for coeliac disease and related terms	*Gut*	2013	62	43	52	790	3
Rubio-Tapia *et al*[41], 2013	ACG Clinical Guidelines: Diagnosis and Management of Celiac Disease	*American Journal of Gastroenterology*	2013	108	656	676	788	3
Hill *et al*[35], 2005	Guideline for the diagnosis and treatment of celiac disease in children: Recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition	*Journal of Pediatric Gastroenterology and Nutrition*	2005	40	1	19	752	4
2001-2005								
Guralnik *et al*[20], 2004	Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia	*Blood*	2004	104	2263	2268	793	1
Hill *et al*[35], 2005	Guideline for the diagnosis and treatment of celiac disease in children: Recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition	*Journal of Pediatric Gastroenterology and Nutrition*	2005	40	1	19	752	4
Grantham-McGregor and Ani[23], 2001	A review of studies on the effect of iron deficiency on cognitive development in children	*Journal of Nutrition*	2001	131	649S	666S	736	1
Pennazio *et al*[33], 2004	Outcome of patients with obscure gastrointestinal bleeding after capsule endoscopy: Report of 100 consecutive cases	*Gastroenterology*	2004	126	643	653	688	3
Nicolas *et al*[28], 2002	Severe iron deficiency anemia in transgenic mice expressing liver hepcidin	*Proceedings of The National Academy of Sciences of The United States of America*	2002	99	4596	4601	694	2
Haas and Brownlie[2], 2001	Iron deficiency and reduced work capacity: A critical review of the research to determine a causal relationship	*Journal of nutrition*	2001	131	676S	688S	573	1
Dubé *et al*[22], 2005	The prevalence of celiac disease in average-risk and at-risk Western European populations: A systematic review	*Gastroenterology*	2005	128	S57	S67	427	1
Ohgami *et al*[31], 2005	Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells	*Nature Genetics*	2005	37	1264	1269	394	2

Beard and Connor[24], 2003	Iron status and neural functioning	*Annual Review of Nutrition*	2003	23	41	58	383	1
Graham *et al*[37], 2005	Visible small-intestinal mucosal injury in chronic NSAID users	*Clinical Gastroenterology and Hepatology*	2005	3	55	59	391	4
2006-2010								
Malfertheiner *et al*[34], 2007	Current concepts in the management of Helicobacter pylori infection: the maastricht III consensus report	*Gut*	2007	56	772	781	1481	3
Bhutta *et al*[44], 2008	Maternal and Child Undernutrition 3 - What works? Interventions for maternal and child undernutrition and survival	*Lancet*	2008	371	417	440	1147	4
Darmon and Drewnowski[19], 2008	Does social class predict diet quality?	*American Journal of Clinical Nutrition*	2008	87	1107	1117	1061	1
Walker *et al*[45], 2007	Child development in developing countries 2 - Child development: risk factors for adverse outcomes in developing countries	*Lancet*	2007	369	145	157	1033	4
Chey and Wong [39], 2007	American college of gastroenterology guideline on the management of Helicobacter pylori infection	*American Journal of Gastroenterology*	2007	102	1808	1825	830	3
Levin and Rocco [38], 2006	KDOQI clinical practice guidelines and clinical practice recommendations for anemia in chronic kidney disease - Foreword	*American Journal of Kidney Diseases*	2006	47	S9	S145	565	4
Lozoff *et al*[25], 2006	Long-lasting neural and behavioral effects of iron deficiency in infancy	*Nutrition Reviews*	2006	64	S34	S43	532	1
Rostom *et al*[46], 2006	American Gastroenterological Association (AGA) Institute Technical Review on the Diagnosis and Management of Celiac Disease	*Gastroenterology*	2006	131	1981	2002	514	3
Ganz *et al*[29], 2008	Immunoassay for Human Serum Hepcidin	*Blood*	2008	112	4292	4297	502	2
Plum *et al*[26], 2010	The Essential Toxin: Impact of Zinc on Human Health	*International Journal of Environmental Research and Public Health*	2010	7	1342	1365	484	5
2011-2015								
Vos *et al*[14], 2012	Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010	*Lancet*	2012	380	2163	2196	4084	1
Ludvigsson *et al* [40], 2013	The Oslo definitions for coeliac disease and related terms	*Gut*	2013	62	43	52	790	3
Rubio-Tapia *et al* [41], 2013	ACG Clinical Guidelines: Diagnosis and Management of Celiac Disease	*American Journal of Gastroenterology*	2013	108	656	676	788	3
Kassebaum *et al* [18], 2014	A systematic analysis of global anemia burden from 1990 to 2010	*Blood*	2014	123	615	624	623	1
Walker *et al*[47], 2011	Child Development 1 Inequality in early childhood: risk and protective factors for early child development	*Lancet*	2011	378	1325	1338	621	1
Camaschella[48], 2015	Iron-Deficiency Anemia	*New England Journal of Medicine*	2015	372	1832	1843	402	Multiple
Chey *et al*[49], 2015	Irritable Bowel Syndrome A Clinical Review	*Jama-journal of The American Medical Association*	2015	313	949	958	378	3
Balarajan *et al* [21], 2011	Anaemia in low-income and middle-income countries	*Lancet*	2011	378	2123	2135	353	1
Pennazio *et al* [33], 2004	Small-bowel capsule endoscopy and device-assisted enteroscopy for diagnosis and treatment of small-bowel disorders: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline	*Endoscopy*	2015	47	352	376	317	3
Goodnough *et al* [42], 2011	Detection, evaluation, and management of preoperative anaemia in the elective orthopaedic surgical patient: NATA guidelines	*British Journal of Anaesthesia*	2011	106	13	22	303	3

2016-2020								
Chey *et al*[50], 2017	ACG Clinical Guideline: Treatment of Helicobacter pylori Infection	*American Journal of Gastroenterology*	2017	112	212	239	324	3
Amieva and Peek [51], 2016	Pathobiology of Helicobacter pylori-Induced Gastric Cancer	*Gastroenterology*	2016	150	64	78	224	4
Kyu *et al*[52], 2016	Global and National Burden of Diseases and Injuries Among Children and Adolescents Between 1990 and 2013 Findings From the Global Burden of Disease 2013 Study	*Jama Pediatrics*	2016	170	267	287	214	1
Girelli *et al*[30], 2005	Hepcidin in the diagnosis of iron disorders	*Blood*	2016	127	2809	2813	147	2
Kassebaum[53], 2016	The Global Burden of Anemia	*Hematology-oncology Clinics of North America*	2016	30	247	308	141	1
Trenkwalder *et al* [54], 2016	Restless legs syndrome associated with major diseases A systematic review and new concept	*Neurology*	2016	86	1336	1343	117	4
Xie *et al*[27], 2016	Advances on Bioactive Polysaccharides from Medicinal Plants	*Critical Reviews in Food Science and Nutrition*	2016	56	S60	S84	112	5
Enns *et al*[55], 2017	Clinical Practice Guidelines for the Use of Video Capsule Endoscopy	*Gastroenterology*	2017	152	497	514	111	3
Froessler *et al*[36], 2018	The Important Role for Intravenous Iron in Perioperative Patient Blood Management in Major Abdominal Surgery A Randomized Controlled Trial	*Annals of Surgery*	2016	264	41	46	98	4
Vasanawala *et al* [56], 2016	Safety and technique of ferumoxytol administration for MRI	*Magnetic Resonance in Medicine*	2016	75	2107	2111	93	3 and 4

For the Topic column, the following numbers correspond to these topics: (1) Epidemiologic aspects of iron deficiency anemia; (2) Biochemical aspects of iron deficiency anemia; (3) Clinical evaluation of causes of iron deficiency anemia; (4) Causes of iron deficiency anemia; and (5) Bioavailability of dietary iron.

Clinical evaluation and management of causes of iron deficiency anemia

Several of the papers in the current study that detailed the causes of iron deficiency anemia were consensus papers that also recommended protocols for the clinical evaluation of patients. These include the American college of Gastroenterology guidelines and the Maastricht III report, which provided guidance on the management of *Helicobacter pylori* infection[34,39], and the Oslo and American College of Gastroenterology guidelines for celiac disease[40,41]. Presurgical workup of anemias was also a highly-cited topic: An example is the NATA guidelines for the evaluation of anemia in preoperative orthopedic patients[42]. The use of perioperative intravenous iron after abdominal surgery has been an important recent contribution to the list of highly-cited papers[36].

Limitations

Bibliometric analysis, although a potent means for highlighting the research hotspots of a given field, has some limitations. First, the bibliometric data are best accessed using the Web of Science database, and currently are not as widely available in other databases. This may cause problems with the search, since checklists for systematic reviews such as PRISMA generally recommend the use of 2 or more databases for searches[43]. Another limitation is that since the number of citations of a given paper would be expected to increase over time, it would be expected that older papers would have a higher citation rate than more recently published papers.In the current study, dividing the study period into 5-year quartiles was an attempt to identify papers which had a high citation rate within their cohort.

CONCLUSION

This study was a bibliometric analysis of the medical literature on iron deficiency anemia published over the last 20 years. Five research hotspots were identified; the area of epidemiology had the largest number of highly cited papers, and the area of bioavailability of dietary iron had the lowest. These relative contributions have been

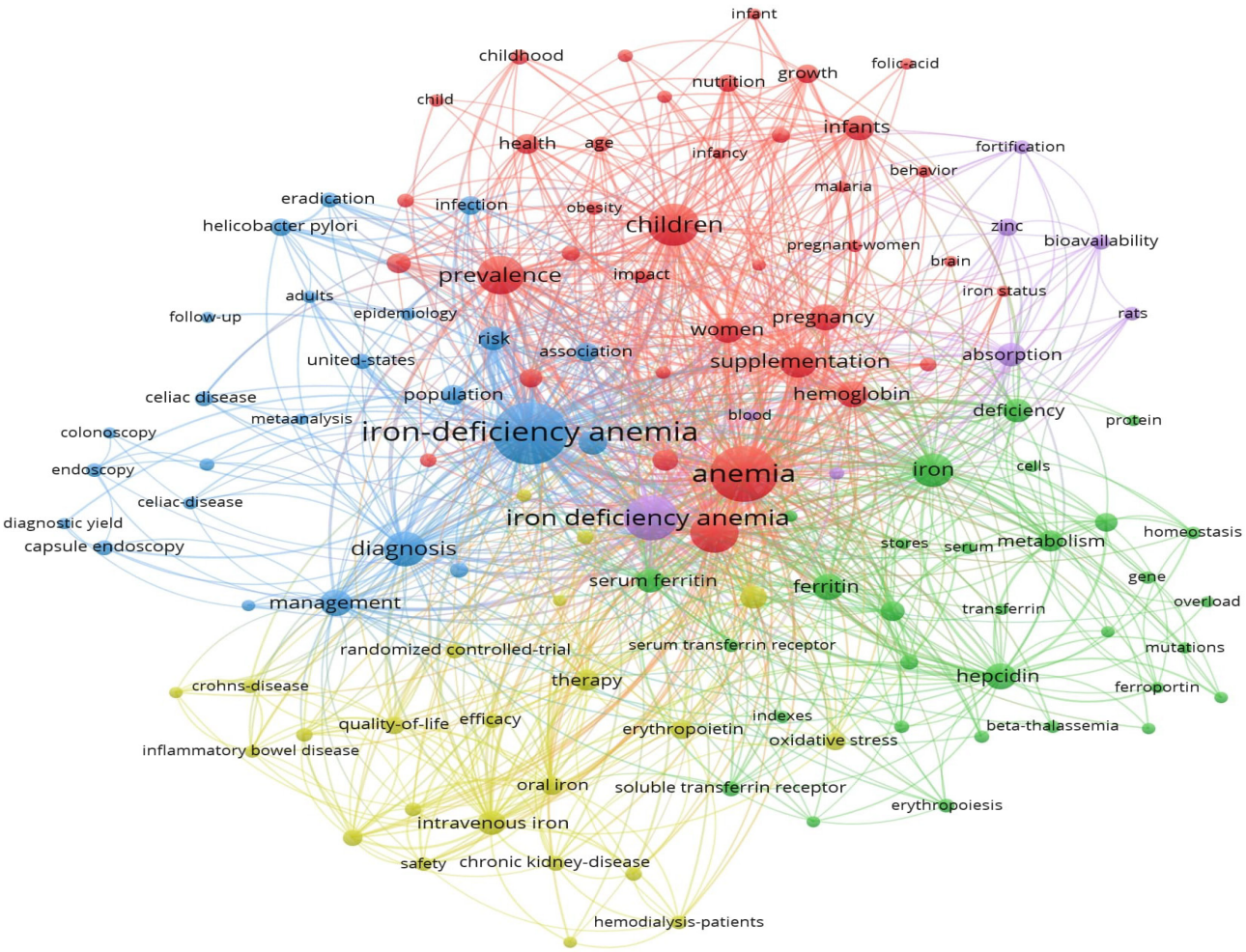


Figure 4 123 terms appearing 50 times or more in the titles or abstracts of the papers in this study.

apparently unchanged over the course of the study period. Although iron deficiency anemia has a worldwide impact, the published research of this period was highly influenced by studies from the United States.

ARTICLE HIGHLIGHTS

Research background

Iron deficiency is the most common micronutrient deficiency and has a worldwide impact. A bibliometric analysis focused on research trends on the topic of iron deficiency anemia has not been performed.

Research motivation

Analyzing the iron deficiency anemia research published between 2001-2020 what collaborative research networks are most active in this field? What subfields are of greatest importance? Are there any emerging research trends in this area?

Research objectives

The objectives of this study were to evaluate the literature published over the last 2 decades, focusing on: (1) The extent of collaborative research networks in this field; (2) The research topics of greatest prominence; and (3) A subset analysis of the most-cited papers published between 2001-2020 and the most-cited papers in 5-year intervals during this period to assess for emerging trends in research in this area.

Research methods

This study was conducted as a bibliometric analysis using Preferred Reporting Items

for Systematic Reviews and Meta-analysis guidelines. A search of the medical literature published between 2001-2020 and related to iron deficiency anemia was performed and data from the search were used to identify publication trends in iron deficiency anemia research. Network visualization by VOSviewer (Leiden University) was performed to identify international collaborative groups and research hotspots in works published during this period.

Research results

Of 4828 publications were included in the study. Network visualization identified 3 international collaborative networks: United States, Canada, and India; Turkey, China, and Japan; and England and other European countries. Five research hotspots were highlighted: (1) Epidemiology of iron deficiency anemia; (2) Biochemistry of iron deficiency anemia; (3) Clinical evaluation of iron deficiency anemia; (4) Causes of iron deficiency anemia; and (5) Bioavailability of iron in the diet. An additional analysis of the top-10 overall cited papers, and the top-10 cited papers for each 5-year increment starting in 2001 showed that the largest number of highly cited papers were from the field of epidemiology, the smallest number from the field of bioavailability of dietary iron.

Research conclusions

Iron deficiency anemia has a high citation rate compared to studies of other topics using similar methodology. Studies from the United States and epidemiologic studies dominate the field.

Research perspectives

Future studies directed from relatively underrepresented areas of the world, and studies directed at less prominently featured areas such as bioavailability of dietary iron may be welcome additions to this already well-developed research area.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
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ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 October 28; 9(5): 405-473



Published by Baishideng Publishing Group Inc

World Journal of Meta-Analysis

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World Journal of Meta-Analysis

Bimonthly Volume 9 Number 5 October 28, 2021

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INDEXING/ABSTRACTING

The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Hua-Ge Yu*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Saurabh Chandan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2308-3840/editorialboard.htm

PUBLICATION DATE
October 28, 2021

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 October 28; 9(5): 405-410

DOI: 10.13105/wjma.v9.i5.405

ISSN 2308-3840 (online)

OPINION REVIEW

COVID-19 and psychiatry training: A cross-national trainee perspective

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Author contributions: Gnanavel S conceptualised and prepared the manuscript; Mathur R, Sharma P, and Parmar A provided valuable inputs and proof read the manuscript before submission.

Conflict-of-interest statement: No conflicts of interest pertaining to this manuscript.

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Manuscript source: Invited manuscript

Specialty type: Psychiatry

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Abstract

The coronavirus disease 2019 pandemic has significantly altered many aspects of our professional lives, including how psychiatry as a medical discipline is taught and learnt. Training in psychiatry relies on developing competencies through observing and interacting with patients, developing empathic consultation skills and seeking feedback from colleagues derived from cognitive and constructivist theories of learning, in a time-bound manner. The pandemic has drawn attention to the dual role of psychiatry residents as both trainees and physicians, with a pressing identity crisis at an inopportune time. This paper aims to illustrate some of the emerging themes in psychiatry training during the pandemic and some solutions for the same.

Key Words: COVID-19; Psychiatry; Training; Teleconsultation; Review

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Core Tip: There is an urgent need to streamline processes for entry and exit to a psychiatry training program, where it does not exist. Utilizing alternative modes of assessment including anonymized colleague, peer and patient feedback can supplement online assessment tools. Curricular adjustments taking current circumstances into

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: April 20, 2021
Peer-review started: April 20, 2021
First decision: July 14, 2021
Revised: August 6, 2021
Accepted: September 4, 2021
Article in press: September 4, 2021
Published online: October 28, 2021

P-Reviewer: Löffler-Stastka H
S-Editor: Liu M
L-Editor: A
P-Editor: Yu HG

account would be well appreciated by trainees. The most important recommendation we propose is provision of formalised intensive training around teleconsultation skills, using simulated scenarios followed by assessment, in addition to guidelines and modus operandi around remote working.

Citation: Gnanavel S, Mathur R, Sharma P, Parmar A. COVID-19 and psychiatry training: A cross-national trainee perspective. *World J Meta-Anal* 2021; 9(5): 405-410
URL: https://www.wjgnet.com/2308-3840/full/v9/i5/405.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i5.405

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has significantly altered many aspects of our professional lives, including how psychiatry as a medical discipline is taught and learnt. Training in psychiatry relies on developing competencies through observing and interacting with patients, developing empathic consultation skills and seeking feedback from colleagues derived from cognitive and constructivist theories of learning, in a time-bound manner.

The pandemic has drawn attention to the dual role of psychiatry residents as both trainees and physicians, with a pressing identity crisis at an inopportune time. This paper aims to illustrate some of the emerging themes in psychiatry training during the pandemic and some solutions for the same[1].

PROFESSIONAL IDENTITY

Many residents have been expected to assume roles that are not a prescribed part of their specialty training. Further, in some cases residents have also been compelled to acquire skills on the job that were not an expectation when they began residency in psychiatry. While this has resulted in identity crisis in terms of speciality, there is also the additional fear of delay in gaining competencies relevant to psychiatry. Also, due to staff shortages there have been other cases where a premature or an inappropriate delegation of increased responsibility (though within psychiatry) but without the associated privileges including monetary compensation or malpractice indemnity coverage. This results in "Role Confusion", in which a trainee is considered competent to take on a more senior role but actually this may result in a significant delay in their professional development[2]. This is a common theme noted both in both developed and developing countries. In a way, the pandemic has been a masterclass for trainees on practical leadership skills by testing their ability to cope with uncertainty and to make difficult decisions amid heightened anxiety and incomplete information.

CLINICAL SUPERVISION AND DIRECT OBSERVATION

Psychiatry trainees often rely on direct clinical observation and supervision to develop their clinical competencies. This can be an important tool to understanding the complexities of clinical psychiatry. However, this aspect of the training has been significantly compromised during the pandemic. It is currently unclear if virtual means of observation are as effective[3]. However, on a positive note, some residents have reported that the virtual world has opened up and improved lines of communication bringing down the threshold for impromptu teaching or brief ad hoc supervision for specific cases[4].

However, there is a rather unanimous consensus that direct supervision of trainees by psychiatrists has been significantly affected by the pandemic. Trainees seek feedback regarding their interview style, approach, and clinical decision making for which direct observation is imperative. When done virtually, there are inherent problems including difficulty in observing certain subtleties of the mental status examination or non-verbal communication. Also, technological issues like weak internet signals and poor video/audio can compromise on the quality of the

interaction[3]. Similarly, wearing personal protective equipment (PPE) adds a physical barrier between trainees and patients that often obstructs observation of facial expressions that can be crucial for a quality assessment[5].

While most of the clinical care transitioned to the virtual format during the pandemic in developed countries, it was more of a challenge in developing countries due to the 'digital divide' characterised by lack of access to quality internet[6]. This also meant that psychiatric training in developing countries was disproportionately affected during the pandemic. Softening of regulations involving tele-mental health *e.g.*, Health Insurance Portability and Accountability Act, 1996 in United States have significantly expanded the scope of virtual care provisions, particularly in developed countries[7].

Also, most of the mental health services in public sector are through teaching hospitals in tertiary centres in developing countries (including South Asia) through self-referral unlike the tiered mental health provisions that exist in developed countries like United Kingdom. Hence, COVID related factors including lock downs and social restrictions are likely to have a disproportionate impact on our patients in developing countries. This also translates to a corresponding disproportionate impact on psychiatric training in developing countries due to the significantly reduced foot fall in tertiary psychiatric institutions, typically located in metro cities (during lockdown periods).

Previous literature emphasises how personalized feedback systems facilitate change. The more subjective perception and experience is assessed and reconsidered, the more significant change can actually take place. Differential steps may be considered to promote motivation, provide more security in disruptive times, and make change possible while supervising trainees. Triangulated research designs and domain knowledge can be considered together with an idiographic assessment of subjective values, subliminal affect perceptions, attitudes, values and beliefs to better facilitate this process[8].

CURRICULAR CHANGES INCLUDING ASSESSMENTS:

In United Kingdom, the Royal college of Psychiatry (RCPsych) has been actively working with the trainees to ensure the quality of training is not significantly compromised and are provided the right support with appropriate leeway (in terms of curricular adjustment) at the same time. With most conferences being cancelled, RCPsych has successfully trialed a free webinar series by experts on different topics including but not limited to COVID-19 related topics. A variety of other organisations have also run several webinars useful for psychiatry trainees. Similar examples from developing countries include different divisions of Indian psychiatric society (IPS) and Psychiatrists association of Nepal (PAN) have been actively running webinar and virtual sessions, more aimed at trainee psychiatrists that have been well received. The local teaching programs have transitioned online in most institutions in all the three countries. In fact, an off shoot of this has been some well received local teaching sessions are now rolled out virtually across institutes or even different nations globally. In United States, the National Neuroscience Curriculum Initiative produced a "quarantine curriculum", which has been well-received by trainees, including lectures ranging from complex trauma, borderline personality disorder to child psychiatry and psychosis[9].

In United Kingdom, at a national level, examinations, including Clinical assessment of skills and competencies (CASC) and appraisal have all moved to virtual platforms [10]. The online CASC exam successfully rolled out recently was possibly the first of its kind globally. Standardized role players and simulation techniques have been successfully utilized for these exams. Plenty of thought and consideration has been provided to provide leeway to trainees in terms of career progression including reduction in number of work-place assessments (WPBA) needed and encouraging pieces of self-reflection (including on coping with complex work environment in the background of COVID-19) in portfolio to compensate in lieu of WPBA's.

Similarly, IPS and PAN recommend utilizing virtual platforms for assessment and examinations. However, it is concerning to note a persisting ambiguity in decisions on entry/exit exams in different institutions in South Asia. It is even more concerning to note a possible delay in completion of the training period for trainees in some of the institutions that results in increase in apprehension among trainees.

Previous literature demonstrates how synergistically combining textbook, e-learning cases and a simulated patient course in psychiatry education can be achieved

using a trans-disciplinary case-based blended learning framework. The same framework can be used to conceptualise assessments in mental health competencies as well[11]. The added advantage is that this can be helpful for curricular development and harmonization with corresponding mental health curricula in other institutes/countries. E-case based blended learning approaches can expedite the transfer of declarative knowledge to procedural knowledge in practice *via* fostering the understanding of pathophysiological concepts[12]. Similarly, standardized patients have been successfully utilized in medical education to train students' communication skills and this paradigm can be successfully employed for the purpose of assessments as well[13].

RESEARCH

Psychiatry trainees across the globe are encouraged to utilize this period for honing theoretical research skills and to consider taking up literature reviews on topics of interest in lieu of ongoing research that has been stalled. However, it is worth noting that some of the institutions traditionally mandate a piece of original research (not literature reviews) as a prerequisite for completion of even general psychiatric training. Those trainees who have been the most affected in this regard are those who have already embarked on research that requires patient contact. Hence, exploring other research projects at this critical juncture is proving increasingly stressful for these trainees[14]. In institutes where case series can be considered as research activity, facilitating e-collection of academic cases with a step wise feedback system provides a dynamic platform to link recent basic research to clinical practice and familiarizing students with research questions and the current research approach[15].

PERSONAL WELL- BEING AND PASTORAL SUPPORT:

In United Kingdom, it is heartening to note a number of trainee well-being initiatives including the psychiatrist support service, support from psychiatrists trainee committee and a number of local/regional initiatives including mindfulness-based sessions, pastoral support and peer support. Similarly, IPS and PAN have come out with resources and initiatives to support trainee psychiatrists. This is crucial when viewed from the lens of Abraham Maslow's 'hierarchy of needs' since the basic needs including safety need to be met first before any higher order needs including educational/training needs[16].

This approach also included education about good hygiene habits to prevent cross-contamination, access to PPE, surge planning throughout the health system, childcare arrangement, and housing in case of sickness or quarantine. The most helpful interventions were specific or targeted as opposed to general reassurances. For example, sharing institutional dashboards relating the number of occupied beds, number of COVID-related admissions, and detailed contingency plans helped in maintaining a steady flow of accurate information in a transparent manner.

RECOMMENDATIONS

There is an urgent need to streamline processes for entry and exit to a psychiatry training program, where it does not exist. Utilizing alternative modes of assessment including anonymized colleague, peer and patient feedback can supplement online assessment tools. Past interim assessments can also be used to project the final outcome. Simulated role plays or objective structured clinical examination conducted virtually may be a good method for summative assessment. Trainee's research competencies need to be evaluated considering the unprecedented circumstances, to prevent any unfair disadvantage. Curricular adjustments taking current circumstances into account would be well appreciated by trainees. Standard operating procedures need to be chalked out for the current circumstances to contain trainee's anxiety and apprehension on new modes of working.

The most important recommendation we propose is provision of formalised intensive training around teleconsultation skills, using simulated scenarios followed by assessment, in addition to guidelines and modus operandi around remote working. Involving trainee psychiatrists as active stake holders in the entire process from

consultation to implementation as well as providing a uniform and consistent message is likely to significantly improve trainee confidence. Also, making clear the roles and expectations of trainee psychiatrists through open and honest discussions at an individual and collective (organisational) level would be the way forward to allay any anxiety[17].

CONCLUSION

The most important recommendation we propose is provision of formalised intensive training around teleconsultation skills, using simulated scenarios followed by assessment, in addition to guidelines and modus operandi around remote working. Involving trainee psychiatrists as active stake holders in the entire process from consultation to implementation as well as providing a uniform and consistent message is likely to significantly improve trainee confidence. Also, making clear the roles and expectations of trainee psychiatrists through open and honest discussions at an individual and collective (organisational) level would be the way forward to allay any anxiety.

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World J Meta-Anal 2021 October 28; 9(5): 411-437

DOI: 10.13105/wjma.v9.i5.411

ISSN 2308-3840 (online)

REVIEW

Current and future of anterior cruciate ligament reconstruction techniques

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Author contributions: Takahashi T performed and wrote the paper; Watanabe S and Ito T performed the collected the references.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Manuscript source: Invited manuscript

Specialty type: Orthopedics

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

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Abstract

In recent years, anterior cruciate ligament (ACL) reconstruction has generally yielded favorable outcomes. However, ACL reconstruction has not provided satisfactory results in terms of the rate of returning to sports and prevention of osteoarthritis (OA) progression. In this paper, we outline current techniques for ACL reconstruction such as graft materials, double-bundle or single-bundle reconstruction, femoral tunnel drilling, all-inside technique, graft fixation, preservation of remnant, anterolateral ligament reconstruction, ACL repair, revision surgery, treatment for ACL injury with OA and problems, and discuss expected future trends. To enable many more orthopedic surgeons to achieve excellent ACL reconstruction outcomes with less invasive surgery, further studies aimed at improving surgical techniques are warranted. Further development of biological augmentation and robotic surgery technologies for ACL reconstruction is also required.

Key Words: Anterior cruciate ligament reconstruction; Surgical techniques; Revision surgery; Biological augmentation; Computer-aided surgery

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Core Tip: Although anterior cruciate ligament (ACL) reconstruction has offered great benefits, particularly to athletes and physical laborers, there is a great deal of room for

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: March 31, 2021
Peer-review started: March 31, 2021
First decision: July 28, 2021
Revised: August 13, 2021
Accepted: September 10, 2021
Article in press: September 10, 2021
Published online: October 28, 2021

P-Reviewer: Liu L
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M



improvement through technology development aimed at achieving more excellent outcomes and restoring performance to a level equal to or higher than before the injury. The all-inside ACL reconstruction technique is a relatively new, minimally invasive method in which both femoral and tibial tunnels are drilled from inside the joint, and its advantages include less postoperative pain and less bleeding. A new computer-aided ACL reconstruction system with high efficacy needs to be developed.

Citation: Takahashi T, Watanabe S, Ito T. Current and future of anterior cruciate ligament reconstruction techniques. *World J Meta-Anal* 2021; 9(5): 411-437
URL: https://www.wjgnet.com/2308-3840/full/v9/i5/411.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i5.411

INTRODUCTION

The anterior cruciate ligament (ACL), located in the middle of the knee joint, is significantly associated with the stability of the knee. Numerous studies have contributed to the advancement of knowledge and treatment of the ACL, as well as knee surgery. Given the anatomical location and roles of the ACL, the strategies for treating it, and the approaches to researching it, one could say that "all roads of the knee lead to the ACL". This article discusses current and future trends of surgical treatment for ACL.

Based on the challenges that our predecessors faced, ACL repair is rarely indicated for ACL injury. Instead, ACL reconstruction is usually performed.

In recent years, ACL reconstruction has generally yielded favorable outcomes. This procedure is a minimally invasive arthroscopic surgery involving small incisions, and rarely causes significant complications[1,2]. Therefore, ACL reconstruction has been actively performed for in more and more patients, including young professional athletes as well as middle-aged and older amateur athletes[3-5]. Currently, the number of ACL reconstruction procedures performed in the United States is approximately 200000 per year and is expected to increase further[6,7]. Considering the increasing level of orthopedic treatment in the world, as well as an increase in the number of people who desire a high quality of life that permits a high level of activity, there is no doubt that the number of ACL reconstruction procedures will increase.

Nevertheless, ACL reconstruction requires about 6 mo to return to sport after surgery. Consequently, high school and college athletes, as well as professional athletes, often end up stepping down as a player without a complete comeback after undergoing ACL reconstruction because their time as a player is limited. Even after returning to play, some athletes who have undergone the surgery cannot perform as they did previously because a sense of knee instability remains and the muscle strength does not sufficiently recover. The causes of these unsatisfactory outcomes include the complication of meniscal injury[8], time from injury to reconstruction surgery, progression of osteoarthritis (OA), weakness of the quadriceps, joint laxity such as hyperextended knee, and anatomical characteristics such as excessive posterior tibial slope together with narrow intercondylar fossa[9].

The common causes of ACL reconstruction failure include new traumatic events (38%), technical failure (22%), and combined causes (19%)[10]. Anatomical ACL reconstruction can reduce the risk of post-traumatic OA[11]. Factors associated with postoperative outcome include drill hole position, method of fixation, mechanical strength of the tendon graft, drilling method, whether the ACL remnant is preserved, pediatric patients before epiphyseal closure and treatment of the meniscus[12].

Because ACL reconstruction enables athletes to return to sports and also people of working age (15–65 years old) to return to work[13], it can contribute to maintaining and activating industries in their communities. Although ACL reconstruction has offered great benefits, particularly to athletes and physical laborers, there is a great deal of room for improvement through technology development aimed at achieving more excellent outcomes and restoring performance to a level equal to or higher than before the injury.

In this paper, we outline current techniques for ACL reconstruction, describe their features and problems, and discuss expected future trends.

SURGICAL TECHNIQUES

Graft materials

Preparation: Hamstrings: The hamstring tendon is harvested by making a 3–4 cm skin incision 2 cm distal to the medial tibial articular surface. First, the sartorius tendon is divided along the length of the tendon. The underlying gracilis tendon is confirmed and pulled proximally. The semitendinosus tendon is confirmed distally. The distal branch of the semitendinosus tendon is dissected, bluntly detached with forceps and a gauze ball, and collected using a tendon stripper. If the semitendinosus tendon is short or thin, the gracilis tendon is also harvested.

Bone–patellar tendon–bone: The bone–patellar tendon–bone (BTB) graft is harvested by making a longitudinal skin incision of approximately 5 cm along the medial edge of the patellar tendon. An incision is made in the central 9–10 mm of the width of the patellar tendon with a scalpel. Trapezoidal bone fragments with a width of 8–10 mm and a length of 15 mm are collected from the patella and tibial tuberosity. In a BTB graft, when the length of the tibial tunnel is short and the patellar tendon is long, the bone fragment on the tibial side is exposed outside of the tibial bone tunnel. Thus, it is necessary to prepare a fixture such as a post screw.

Quadriceps: The surface layer of the quadriceps is the rectus femoris tendon. The middle layer consists of the tendons of the vastus medialis and vastus lateralis muscles. The deep layer is the vastus intermedius tendon. The width is narrowest approximately 5 cm proximal to the patellar attachment[14]. To harvest the quadriceps tendon, a scalpel is used to make a full-thickness incision 5–6 mm from the patellar attachment to the proximal end of harvested tendon. Next, an incision is extended up to the patellar attachment along the length of the tendon. The width of adherent portion is 8–10 mm. Next, a tendon with a thickness of approximately 10 mm is harvested. A Krackow suture is performed with two sets of No. 2 sutures. The length of the grafted tendon can be easily adjusted.

On the patellar side, a trapezoidal bone fragment with a length of 15 mm and a width of 8–10 mm is harvested. Even if the width of the quadriceps tendon is 5–6 mm, the thickness (approximately 10 mm) is sufficient in combination with the rectus femoris, vastus medialis, vastus lateralis, and vastus intermedius. The cross-sectional area of the quadriceps tendon is almost twice that of the BTB graft. The quadriceps tendon has higher load to failure and stiffness than the BTB graft[15]. The quadriceps tendons may be easier to use than the BTB graft in patients with anterior knee pain and pain during kneeling[16]. To fix the grafted ligament, an interference screw or a cortical button such as the CL-BTB is used as a patellar fragment on the femoral side. The thread is tied tightly to the cortical button on the tibial side.

Hamstring and BTB is commonly used as autografts[17]. Several studies reported no significant difference between these materials in postoperative clinical outcome; knee stability evaluated by KT-1000 (MED metric, San Diego, CA) test, Lachman test, and/or pivot shift test; International Knee Documentation Committee (IKDC) score; knee injury and osteoarthritis outcome (KOOS) score; limitation in range of motion; or the rate of return to sports[18-21]. However, there are some reported that the rate of graft rupture was slightly higher in patients who used hamstring autograft than in those who used BTB autograft[22-24] (Table 1).

Characteristics: Hamstrings: Among the hamstrings, the semitendinosus tendon and gracilis tendon are most commonly used for ACL reconstruction in patients, including amateur athletes[25]. If possible, it is preferable to harvest only the semitendinosus tendon, in order to prevent postoperative muscle weakness[26]. For single-bundle ACL reconstruction, the semitendinosus tendon is folded into four layers to obtain a diameter ≥ 8 mm. If sufficient length or diameter of autograft cannot be obtained, there is no choice but to use the gracilis tendon. A possible solution when performing ACL reconstruction using only the semitendinosus tendon autograft is to fill the socket-like drill tunnels with graft and use a technique with fewer bungee cord and windshield wiper effects. Harvesting the hamstring may result in reduced mobility of the knees at a high flexion position in some cases such as ballet dancer. An advantage of ACL reconstruction with hamstring autograft is that it makes it easy to perform double-bundle ACL reconstruction (Table 1).

BTB: ACL reconstruction with BTB autograft has commonly been used around the world because the patellar tendon provides high mechanical strength and interference screws provide strong fixation. However, because the autograft is harvested with bone, this technique causes postoperative tenderness of the anterior knee region where harvest was performed. This pain can persist for several years. Accordingly, the use of BTB autograft might be avoided for people in some Asian countries where it is

Table 1 Characteristics of various autograft materials

	Hamstrings	BTB	Quadriceps
Cross-sectional area	Good to excellent	Fair to good	Good to excellent
Mechanical strength	Good	Good to excellent	Excellent
Adjustment of graft length	Possible	Difficult	Easy
All-inside technology	Easy	Sometimes difficult	Easy
Preservation of remnant	Possible	Sometimes difficult	Sometimes difficult
Double bundle	Easy	Difficult	Possible
Graft fixation			
Femoral	Cortical button	Interference screw	Interference screw
		(metalic > bioabsorbable)	(metalic > bioabsorbable)
		Cortical button	Cortical button
Tibial	Cortical button	Interference screw	Cortical button
	Interference screw	(metallic ≥ bioabsorbable)	Interference screw
	(metalic < bioabsorbable)		
	Post fixation		
Complication	Nerve injury	Patellar fracture	Patellar fracture
	(infra-patellar branches of the saphenous nerve)		
	Decrease of Flexor muscle strength	Kneeling pain	
		Anterior knee pain	
		Decrease of extensor muscle strength	Decrease of extensor muscle strength
Indication			
Recommend	Amateur athlete	Amateur and professional athlete	Amateur and professional athlete
		(high-intensity sports)	(high-intensity sports)
			Revision surgery
Not recommend	Ballet dancer	Wrestler, Judo, Karate	

BTB: Bone–patellar tendon–bone.

common to sit on the Japanese sitting or kneeling and for athletes that requires often kneeling, such as wrestler judo or karate. Even in these countries, however, BTB autograft is often considered to be the first choice in male patients who do high-intensity sports because it can provide better postoperative stability relative to ACL reconstruction with hamstring autograft[21,27,28] (Table 1).

Quadriceps: Reports on the use of quadriceps tendon autograft with or without a bone block have increased since 2015, although the method had been used previously in clinical practice. Some studies reported that the clinical outcomes of ACL reconstruction with quadriceps tendon autograft were comparable or superior to those of reconstruction with BTB or hamstring autograft[28-31], whereas other studies reported that the rate of graft rupture was higher in ACL reconstruction with quadriceps tendon autograft than in reconstruction with BTB or hamstring autograft [32]. ACL reconstruction with quadriceps tendon autograft has several advantages: it causes less pain in the anterior knee region because there is a bone plug on one side only[29,33], less risk of injury to the infrasaphenous branch and the procedure requires only a small skin incision[34]. By contrast, ACL reconstruction with BTB autograft damages the tibial tuberosity. Although several studies reported ACL reconstruction with quadriceps tendon autograft, most were based on short-term follow-up. Hence, further studies based on long-term follow-up are needed to explore the possibility of reduced muscle strength for knee extension associated with the procedure (Table 1).

ACL reconstruction with BTB or quadriceps tendon autograft temporary decreases muscle strength for knee extension because it damages the knee extensor mechanism.

In addition, reduced quadriceps strength may be exacerbate knee OA. Therefore, sufficient training for these muscles is required after surgery.

Allograft: ACL reconstruction with allograft is an alternative technique because it does not damage the patient's own tissues. Therefore, it has been performed widely in the United States and Europe, and its outcomes are comparable to those of ACL reconstruction with autograft. However, after a study reported that the rate of graft rupture in patients who underwent ACL reconstruction with allograft was higher than that in those who received autograft[35,36], the use of allograft for primary ACL reconstruction has gradually decreased, although ACL reconstruction with fresh frozen and non-irradiated allograft is sometimes performed for revision ACL reconstruction[37,38] and multiligament reconstruction. The use of allograft is generally not recommended for primary ACL reconstruction in elite athletes.

Techniques for femoral tunnel drilling

The most common technical error in ACL reconstruction procedure is femoral tunnel malposition (63%), which causes poor clinical outcome due to residual instability or graft rupture[39].

Independent drilling technique is a method for drilling femoral and tibial tunnels separately (Table 2). There are two types of independent drilling: 1) the anteromedial (transportal) technique, in which a femoral tunnel is drilled from the inside to the outside; and 2) the outside-in technique, in which a femoral tunnel is drilled from the outside to the inside on a footprint identified with a dedicated drill guide (Table 1).

Anteromedial technique: The arthroscope is inserted from the anterolateral portal with the knee bent to 120–130 degrees. The drill guide pin is inserted from the anteromedial portal. The pin tip is placed in the center of the femoral footprint of the ACL. After drilling, it penetrates from the lateral cortex to the skin surface. First, a PL bundle bone tunnel is made, followed by an AM bundle bone tunnel in the same manner. If the knee flexion angle is 120 degrees or less, blowout of the posterolateral cortex would occur[40-42], making it impossible to fix the femoral side; this increases the risk of peroneal nerve palsy.

Outside-in technique: The arthroscope is inserted from the anteromedial portal with the knee bent to approximately 90 degrees. The femoral drill guide is inserted into the joint from the anterolateral portal and held firmly in place. Next, the guide pin is inserted so that it does not shift from the center of the femoral footprint of the ACL. The drill-guided trocar should be placed on the femur. A skin incision should be made in the lateral thigh to locate the lateral cortex.

With the anteromedial and outside-in approaches, after creating the femoral bone tunnel, a tibial drill guide is used to create a tibial bone tunnel from the anterior surface of the tibia based on the bone tunnel diameter. Both techniques are useful in that they enable surgeons to accurately create a femoral tunnel on the target footprint. Although both techniques allow for accurate femoral tunnel positioning, some studies have reported that the outside-in technique is more effective because it results in a more oblique tunnel and a longer femoral tunnel relative to the anteromedial technique[43-45]. Disadvantages of the anteromedial technique include that it is associated with the risk of short femoral tunnel, posterior-wall blowout, and iatrogenic damage to the cartilage of the medial femoral condyle by a more horizontal direction of the femoral tunnel in Three-dimensional (3D) plane[41]. However, the anteromedial technique can also produce a long femoral tunnel by using a flexible reamer, and the risk of peroneal nerve paralysis associated with this technique can be avoided using a specific procedure[46,47]. Disadvantages of the outside-in technique include the need for small incision in the femur[42]. At present, the anteromedial technique is most commonly used in the world[48,49].

Transtibial technique: This is a classical drilling method for ACL reconstruction, a tibial tunnel is initially created using a tibial drill guide. An arthroscope is inserted from the anterolateral portal. A tibial drill guide is inserted into the joint from the anteromedial portal. The tip of the guide is applied to the footprint of the tibia and then a guide pin is inserted through the tibial tunnel to create a femoral tunnel (Table 1). The position of the femoral tunnel in this technique depends on the orientation and position of the tibial tunnel. Therefore, this technique is called dependent drilling. Some studies reported that the anteromedial technique was associated with less femoral tunnel positioning errors and provided better stability and clinical outcomes than the transtibial technique[10,50,51], whereas other studies reported no difference between these techniques in clinical outcomes, patient satisfaction, or rate of revision reconstruction surgery in recreational athletes[52,53].

Table 2 Features of independent and dependent techniques

	Independent technique		Dependent technique		
	Anteromedial	Outside-in	TT	Modified TT	TT with modified devices
Femoral tunnel position	Anatomical	Anatomical	Somewhat unanatomical	Anatomical	Anatomical
Complexity of technique	Relatively simple	Somewhat complicated	Simple	Simple	Simple

TT: Transtibial.

The independent drilling techniques (anteromedial and outside-in) allow for the creation of femoral tunnels at more anatomical positions than the transtibial technique [54]. To decrease the rate of erroneous femoral tunnel positioning with the transtibial technique, several technologies assist in determining the center of the femoral tunnel, *e.g.*, the Wire-navigator® device (a guidewire navigation device; Smith & Nephew Japan Inc., Tokyo, Japan), which is composed of a Navi-tip consisting of tibial and femoral indicators[55] and a laser-beam guided drill guide[56]. These devices can indicate the center of femoral tunnel.

In the modified transtibial technique, the patient's leg is placed in a figure-of-4 position (the knee is in 90° flexion, varus and internal rotation of the tibia, and the hip is abducted) when the guide pin is inserted[57,58]. This technique is easy to perform for many surgeons who are accustomed to the transtibial technique[59]. Some studies reported that this technique resulted in better femoral tunnel positioning than the transtibial technique, as well as femoral positioning and clinical outcomes comparable to those of the independent techniques; in addition, the technique is easy to perform [58,60-62].

In our opinion, it is not ideal to perform the transtibial technique in the classical manner, relying on the surgeon's experience or gut feeling, because in some cases it results in poor femoral tunnel positioning. Therefore, if the transtibial technique is performed, assistive devices such as a dedicated drill guide should be used, and the patient's leg should be placed in a position suitable for this technique. To confirm the femoral tunnel position, some studies have recommended using arthroscopic views through the anteromedial portal as well as intraoperative fluoroscopic views[39].

There are knacks and pitfalls in any of the techniques mentioned above. Therefore, it is important for surgeons to master techniques that they are good at, taking their learning curve into account.

All-inside technique

The all-inside ACL reconstruction technique (Figure 1) is a relatively new, minimally invasive method in which both femoral and tibial tunnels are drilled from inside the joint[63-65]. It has recently become more common in clinical practice, and the number of its case studies has been increasing[66]. The all-inside technique may be a reasonable option. When drilling femoral and tibial tunnels using this technique, special care should be taken not to damage the trabecular bone structure adjacent to the graft and the sutures used to pull the graft. This technique can alleviate postoperative pain because it causes less damage to the tibial bone and the periosteum [67-69]. Moreover, it allows for creation of an autograft using only the semitendinosus tendon by the hamstrings tendons. All-inside ACL reconstruction with a semitendinosus tendon autograft has achieved good postoperative stability of the knee relative to ACL reconstruction with an autograft using both the semitendinosus tendon and the gracilis tendon[70] and ACL reconstruction with BTB[71].

For the all-inside technique, independent drilling with a retrograde drill is commonly performed, and devices that have been modified for this technique are available[68,72,73]. In our hospital, we assemble a dedicated guide pin and reamer in the joint, fix them, and drill the tunnels from inside the joint using the dependent technique[74]. We have achieved good postoperative outcomes in transtibial ACL reconstruction procedures, in which the center of the femoral footprint is irradiated with a laser beam to determine the tunnel position[75]. To date, we also have achieved good postoperative stability in patients who underwent transfemoral all-inside ACL reconstruction.

The advantages of the all-inside technique include less postoperative pain[68] and less bleeding, which may decrease the risk of postoperative infection. If this technique becomes less complicated, more surgeons may choose it (Table 3).

Table 3 Features of the conventional technique and all-inside technique

	Conventional technique	All-inside technique
Invasiveness in the tibial tunnel		
Bone damage	Moderate	Minor
Bleeding	Moderate	Minor
Invasiveness during autograft harvesting	Minor or moderate	Minor
Postoperative pain	Moderate	Minor
Complexity of surgery	Minor	Moderate

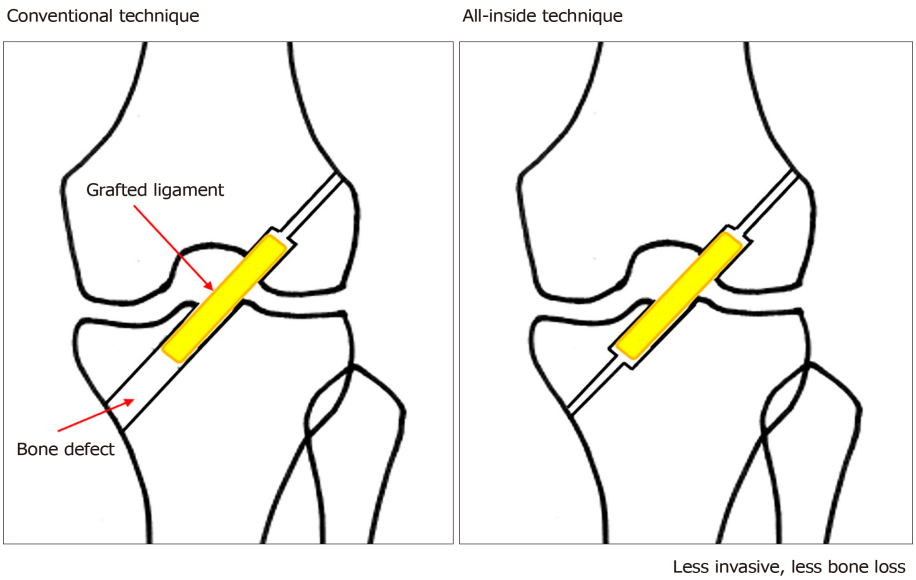


Figure 1 Schema of bone tunnel creation of anterior cruciate ligament reconstruction. Left: Conventional technique; Right: All-inside technique.

In most all-inside ACL reconstruction procedures in which both ends of an autograft are fixed with cortical buttons, only the semitendinosus tendon is used to create the autograft. In such cases, it is possible to create a large-diameter autograft and to obtain a better knee flexion strength in comparison with ACL reconstruction using both the semitendinosus tendon and gracilis tendon[76]. Several studies reported that all-inside ACL reconstruction using adjustable-length loop cortical button fixation resulted in less tibial tunnel widening than ACL reconstruction using bioabsorbable interference screw fixation[77,78], suggesting this approach may avoid two stage revision in revision ACL reconstruction surgery. However, there is no difference between these techniques in postoperative knee stability, clinical outcome, or rate of graft rupture[79]. It is expected that discussion of the benefits and reliability of the all-inside technique will become more active, as at present there have been few reports describing its long-term outcomes (≥ 5 years).

Double-bundle or single-bundle ACL reconstruction

Since the double-bundle ACL reconstruction technique was reported by Muneta *et al* [80] in 1999, it has been used around the world.

A double bundle consists of two routes: AM and PL bundles. Two bone tunnels are created for the femur and tibia, respectively. The graft material consists of hamstring autograft or allograft. In most cases, a hamstring autograft is used. The anteromedial approach is often used to create bone tunnels, but the transtibial or outside-in approach may be used.

An arthroscopic ruler can be used to measure the insertion site of the patient's native ACL. This measurement can help decide whether to perform double- or single-bundle reconstruction[81]. When a patient has femoral and tibial insertion sites that are larger than 14 mm, double-bundle reconstruction is indicated. When a patient has a notch width of less than 12 mm, double-bundle reconstruction often cannot be performed because the AM guide pin cannot be placed in the native femoral insertion

site.

Comparing with single-bundle ACL reconstruction, double-bundle ACL reconstruction can better reproduce the natural anatomical structure of the ACL and provide better ACL function. In addition, several studies reported that double-bundle ACL reconstruction had a lower positive rate in the pivot shift test than that of one-bundle ACL reconstruction[82-84]. Moreover, double-bundle ACL reconstruction is more effective for improving rotatory instability. The rate of revision ACL reconstruction was lower in patients who received double-bundle ACL reconstruction in their primary surgery[85]. In addition, patients who received double-bundle ACL reconstruction exhibited less widening of the bone tunnel diameter, which leads to joint instability of the knee, than those who received single-bundle ACL reconstruction [86].

However, there was no difference between these techniques in the incidence of side-to-side difference evaluated by KT–1000 testing, Lysholm score, KOOS score, or in the rate of graft rupture[87,88]. Specifically, there was no difference in 5-year or longer outcomes between them.

Although the double-bundle ACL reconstruction technique is commonly used in Japan, the single-bundle ACL reconstruction technique is very common in other countries, including the United States. This may be because single route by BTB autograft is performed in many cases in those countries.

The advantages of single-bundle ACL reconstruction with hamstrings autograft include low costs of material fixation: because the autograft is fixed only at two positions, the number of devices required for fixation is half of that required for the double-bundle technique. In addition, surgical time is shorter because fewer bone tunnels need to be created. There is ongoing discussion regarding which technique is better. Therefore, further studies may be necessary to compare the progression of OA, the rate of graft rupture, and knee stability between single- and double-bundle techniques based on long-term follow-up.

ACL graft fixation techniques

Ten to sixteen percent of patients who undergo ACL reconstruction need revision ACL reconstruction due to new traumatic events or poor postoperative outcomes[89]. Therefore, the fixation technique for the primary ACL reconstruction should be selected to avoid challenging or highly-invasive procedures in possible revision ACL reconstruction surgery.

Currently available fixation techniques for ACL reconstruction include one method in which the ligament substance and bone parts are fixed with interference screws, and another method in which an ACL graft with a suture or artificial ligament is fixed with cortical buttons, post screws, or staples and so on.

A literature review study comparing suspensory fixation with interference screw fixation reported that suspensory fixation resulted in less side-to-side difference in KT-1000 measurements, whereas the interference screw exhibited a higher incidence of ligament rupture; however, there was no difference in IKDC scores between the two approaches[72,90].

Suspensory cortical button: The EndoButton is most commonly used for femoral fixation because it allows for easy and strong fixation and achieves favorable long-term outcomes[25,91]. The CL-EndoButton and CL-BTB EndoButton are available as fixed-loop devices. Recently, adjustable-loop devices have been increasingly used in clinical practice. Such devices are believed to be useful for filling gaps in bone tunnels. Some studies that performed comparisons of mechanical strength between fixed-loop and adjustable-loop devices reported that fixed-type loop devices have higher maximum tensile strength with less displacement[77,92-96], whereas other studies reported no difference between them[97-99]. In a clinical study, no difference in KT-1000 arthrometer measurements was observed between the two types of devices[100]. A modified suspension device with higher tensile strength and stiffness that was recently developed makes graft fixation easier under tension[101], and is expected to be used in clinical practice.

Interference screw: Interference screw fixation is most commonly used in ACL reconstruction with BTB because it provides strong fixation[102]. Although many interference screws are made of titanium, which has high biocompatibility, bioabsorbable interference screws are also used in clinical practice. It should be noted that, in some cases, titanium screws implanted into bones may be difficult to remove in revision ACL reconstruction. Interference screw fixation is commonly used for metallic one in femoral side, for absorbable one in tibial side.

Staple: Staples are commonly used to fix an artificial ligament to the bone when both ends of the graft (*i.e.*, the fixation parts inside the bone tunnels) are reinforced with an artificial ligament. Although they provide strong fixation, care should be taken to prevent bone damage that may occur if the cortex of the tibia is vulnerable.

Post-screw: The ACL graft is anchored to the tibia by inserting a post screw with a washer at the distal part of the tibial tunnel. This technique is easy to perform.

Cross-pin: Fixation of an ACL graft with a cross-pin on the femoral side is associated with lower rate of graft rupture[103]. This technique has been used mainly in Europe and the United States, but its frequency has been decreasing.

Double spike plate: The plate is fixed to the tibia by hammering its spikes into the bone under the index tension. Finally, the fixation is completed by inserting a screw [104].

Preservation of ACL remnant

There are two options for preserving the ACL remnant: (1) Selective augmentation of the anteromedial or posterolateral bundle that is partially damaged[105]; and (2) Double-bundle ACL reconstruction while preserving the ACL remnant[106,107].

One advantage of ACL remnant preservation is that mechanoreceptors preserved in ACL remnant and promote angiogenesis. In addition, this approach is associated with less anterior tibial translation and a lower rate of positive pivot-shift test. Despite these advantages, this technique may cause cyclops lesion. However, one study reported no difference in Lachman test, pivot shift test and IKDC score between ACL reconstruction techniques with or without remnant preservation[108]. In general, small scarred bundles of the anteromedial or posterolateral bundle are augmented in ACL reconstruction with remnant preservation. If preservation of the ACL remnant makes it difficult to create bone tunnels at appropriate positions, it is important to remove the remnant.

Anterolateral ligament reconstruction

Claes *et al*[109] named the ligament-like tissue on the lateral margin of the tibial plateau the anterolateral ligament (ALL). ALL reconstruction is an extra-articular procedure that has recently been performed in combination with standard ACL reconstruction[110]. This combination technique achieves favorable outcomes with a low rate of graft failure[111]. The anterolateral complex of the knee contributes to anterolateral rotatory stability as a secondary stabilizer to the ACL[112], although surgical reconstruction of the anterolateral complex may cause constraint of internal rotation of the tibia[113]. Combined ACL and ALL reconstruction are performed mainly in Europe, but its indication is limited to patients who have severe knee instability due to injury of ACL and other combined ligaments, or who have severe knee instability after ACL reconstruction because it is a highly invasive procedure.

A study on the addition of a lateral extra-articular tenodesis (LET) to ACL reconstruction with BTB graft reported no significant differences in long-term outcomes after ACL reconstruction with or without an LET, but LET may increase the risk of lateral compartment OA[114]. Another study reported that ACL reconstruction in combination with LET was associated with a higher risk of tunnel convergence[115].

Current graft options for ALL reconstruction include iliotibial band, gracilis tendon autograft or allograft, and semitendinosus tendon autograft or allograft; fixation angle varies from 0° to 90°[116]. Further prospective studies, such as a randomized control trial, are needed to compare clinical outcomes, indications and fixation techniques between ACL reconstruction with and without ALL reconstruction[116].

ACL repair

ACL repair with suture anchor for patients with avulsion ACL tears[117], as well as ACL repair combined with biologic healing augmentation for patients with incomplete tears[118], achieves successful outcomes.

An ACL repair technique with additional internal bracing was introduced in a recent study[119-122]. A study of this technique based on a short-term follow-up with small sample size reported that its outcomes were comparable to those of ACL reconstruction[123]. ACL repair may be a good treatment option for partial proximal ACL tears and pediatric ACL tears[124,125]. Although a systematic review of contemporary studies revealed no differences between ACL repair and reconstruction with respect to knee stability and the rate of graft rupture, further studies are needed because these studies were based on short-term follow-up with small sample size[123].

Complication

Infection and its prevention: The incidence of knee joint infection after ACL reconstruction is low because it is performed arthroscopically with saline irrigation [126]. However, infection has been reported to occur in 0.14% to 2.6 % of patients who undergo ACL reconstruction[7,127,128]. The most common pathogen of infection after ACL reconstruction is *Staphylococcus aureus*. Acute infection can be caused by pathogen contamination of the tibial tunnel or the skin incisions made for arthroscopy [126]. Pre-soaking hamstring autografts in gentamicin reduce intra-articular infection rates[129]. Bleeding and subcutaneous hematoma of these sites after surgery can also be a cause of infection. Therefore, it is important to decrease the amount of bleeding by icing and to treat wounds carefully. On the other hand, chronic infection can be caused by screws and tendon suture materials[126]. Special care should be taken for patients with atopic dermatitis, as these patients have a higher infection risk.

Deep vein thrombosis: The incidence of deep vein thrombosis after ACL reconstruction ranges from 0.3%[130] to 0.4%[131]. The incidence of pulmonary embolism is 0.18%[130] to 0.046%[130]. The only significant risk factor is age. Therefore, thromboprophylaxis should be considered in older patients.

Hemarthrosis: Hemarthrosis after ACL reconstruction can delay rehabilitation. The use of intravenous tranexamic acid in ACL reconstruction results in reduced joint drain output and hemarthrosis as well as less pain and greater range of motion during the early postoperative period[132]. Tranexamic acid does not increase the risk of deep vein thrombosis after surgery[133].

Joint stiffness: The incidence of joint stiffness after ACL reconstruction is overall 3% [134] to 8.8%[135]. There was no significant difference between BTB graft and hamstring tendon with respect to the frequency[135] and the interval between trauma to surgery[134].

Cyclops syndrome after ACL reconstruction is due to a fibrous nodule in the anterior part of the intercondylar notch. It restricts the full extension of the knee[136]. The incidence of symptomatic cyclops syndrome ranges from 1.9% to 10.9%[136].

Arthrofibrosis is a rare but potentially devastating complication after ACL reconstruction[7]. Approximately 2% of patients have postoperative stiffness that requires intervention[137]. However, arthrofibrosis remains poorly defined and there are no clear treatment guidelines[138].

Nerve injury: Tendon harvesting for ACL reconstruction often injures sensory branches of the saphenous nerve[139]. Injuries to the sartorial branch of the saphenous nerve associated with medial incisions for hamstring tendon harvesting are more common than injuries to the infrapatellar branch associated with midline incisions for patellar tendon harvesting[139]. Numbness of the skin surface supplied by the infrapatellar branches of the saphenous nerve after ACL reconstruction are less common with the quadriceps tendon compared with the hamstring tendon[140]. Regarding hamstring tendon harvesting for ACL reconstruction, vertical incisions increase the risk of iatrogenic injury to the IPBNS compared with oblique incisions[141-144].

Patellar fracture: The incidence of patellar fracture during BTB harvesting ranges from 0.3%[135] to 1.3%[145]. It is a rare but serious complication[146]. To eliminate the risk of perioperative patellar fracture, the bone-tendon-autograft technique, which does not harvest the inferior patellar bone, might be an alternative graft option[147].

The incidence of intraoperative patellar fracture after harvest of a quadriceps tendon autograft is reported to be 3.5%. It is necessary to use care when harvesting the bone block from a central position[148] and to limit the depth of bone harvesting to less than 50% of the depth of the patella with a shorter bone plug length. Longitudinal cuts can be angled centrally to produce a trapezoidal bone block with shallower bone removal[148].

Our methods for ACL reconstruction

We usually use only the semitendinosus tendon for primary ACL reconstruction. We produce the bone tunnel with the all-inside method using a single quadruped semitendinosus graft. We fix the grafted ligament with a cortical suspension button on both sides. In the all-inside method, the knee is flexed to approximately 90 degrees, the lower leg is internally rotated, and varus stress is applied to the knee using the dependent method.

We developed a tibial drill guide with a laser beam that can identify the optimal location for the femoral tunnel during creation of tibial tunnel in a modified transtibial method. We used it in a clinical application during ACL reconstruction.

The new drill guide system: The structure of the tibial drill guide with a laser beam is shown in Figure 2. This laser beam-guided technique with a special tibial drill guide produces both tibial and femoral tunnels. The laser pointer was visible light semiconductor laser, maximum output energy of 1mW. The guide contains a metal tube for passage of laser beam (Figure 3), which can be filled with saline for irrigation. The reflected beam indicates appropriate position on the extension of the pin (Figure 4). Figure 5 shows an arthroscopic photography with the laser beam.

Transtibial guide pin placement and tunnel placement: The special drill guide is inserted through the anteromedial portal, and placed at the anatomical tibial foot print. A laser beam is reflected by reflecting plate of tip of the guide. The laser pointer illuminates the tunnel which is where femoral bundle should be made appropriately (Figure 4). A transtibial guide pin of 2.4 mm in diameter is inserted into the intra-articular portion of proximal tibia. The diameter of tibial tunnel is similar to that of grafted tendon. The guide pin is set at appropriate location of femoral tunnel. Method of making femoral tunnel and graft fixation was performed according to our previously described[56,75]. Our method is a useful way to select an appropriate anatomical site for the bone tunnels accurately and obtain excellent clinical results with ACL reconstruction.

Recently, we have produced and used a drill guide for the all-inside transfemoral method. The grafted ligaments are fixed with a CL-BTB endobutton on the femoral side and a cortical button on the tibial side with knee flexion of approximately 20 degrees. We often use a BTB graft to obtain strong fixation for young men who are active athletes needing to withstand strong collisions, such as in rugby and football. However, the quadriceps tendon is also useful. Residual ligaments are often preserved if they are thin but relatively tense. At this time, double-bundle reconstruction with residual ligaments is not performed because it is difficult to make two bone tunnels at appropriate positions. We reconstruct the AM or PL bundle based on preoperative MRI evaluation and intraoperative arthroscopic findings. ACL reinforcement is often performed to reconstruct the PL bundle, which can lead to definite symptoms of rotatory instability.

In revision reconstructive surgery, we use the ipsilateral semitendinosus tendon, BTB graft, or quadriceps tendon with a patellar fragment, unless the tendon has already been used. Quadriceps tendon with a patellar fragment has excellent mechanical strength. Reconstruction can be performed with the all-inside method, which reduces trabecular damage in the bone tunnel. Thus, we plan to increase its use in the future. At this time, the grafted ligament is fixed with a patellar fragment on the femoral side using an interference screw. On the tibial side, the grafted ligament is fixed with a cortical button, which is sutured using the Krackow method with two sets of no. 2 sutures.

REVISION ACL RECONSTRUCTION

Some patients may need to receive revision ACL reconstruction due to graft rupture or residual knee instability.

One-stage or two-stage revision ACL reconstruction

When an ACL graft cannot be fixed at an appropriate position because the bone is severely damaged, bone tunnel grafting with an Iliac bone autograft is performed in two-stage revision ACL reconstruction[149]. In most cases, however, one-stage revision ACL reconstruction is selected because the two-stage procedure requires time for healing after bone grafting.

Techniques for revision ACL reconstruction

Prior to revision ACL reconstruction, computed tomography scanning is performed to three-dimensionally assess the positions and sizes of bone tunnels created in the primary surgery and to determine the positions of bone tunnels for revision ACL reconstruction. During the assessment, it is important to confirm the type of fixture used in the primary surgery. It should be noted that, if titanium interference screws were used in the primary surgery, it may become more difficult to remove them over

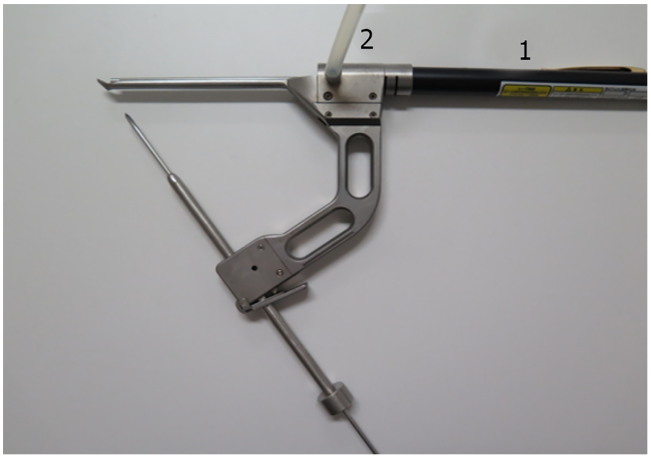


Figure 2 Structure of the tibial drill guide equipment with a laser beam. The laser beam pointer (1); The irrigation tube (2).

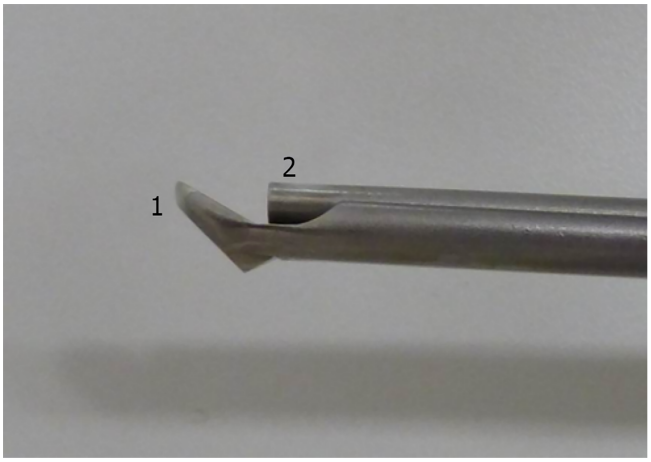


Figure 3 The tip of the tibial drill guide equipment. Reflecting plate (1); Straight metal tube for passage of laser beam (2).



Figure 4 Reflected beam identifying the proper position on the extension of the pin.

time: due to the high biocompatibility of titanium, the screws become surrounded by bone on both femoral and tibial sides. Therefore, appropriate screw drivers should be prepared to remove them. Besides BTB and hamstring grafts, a quadriceps tendon graft is often used in revision ACL reconstruction because it has advantages in terms of strength and diameter[150].

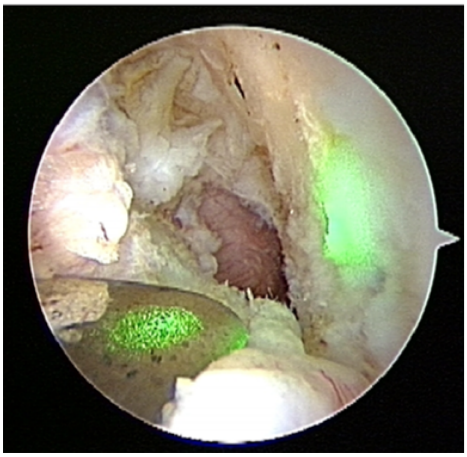


Figure 5 Arthroscopic photography with laser beam. Reflected beam illuminating the foot print of the femoral tunnel.

Postoperative outcomes of revision ACL reconstruction

Several studies reported that revision ACL reconstruction is inferior to primary reconstruction in postoperative outcomes and the rate of returning to sport[151,152]. However, the data regarding long-term clinical outcomes from large-scale cohort studies are limited; accordingly, further studies are needed[150].

TREATMENT FOR ACL INJURY WITH KNEE OA

ACL insufficiency persisting after ACL injury often accelerates age-related OA change, worsens wear of cartilages of the medial knee joint, and results in varus deformity. In this case, ACL reconstruction in combination with high tibial osteotomy (HTO) is indicated for individuals younger than 70 years old who want to engage in a high level of physical activity such as sports or heavy physical labor and have less OA change in the patellofemoral joint[153,154]. Slope-reducing tibial osteotomy with this combination procedure can further improve knee stability in patients with varus deformity and excessive posterior tibial slope[155,156]. There are surgical techniques for HTO include opening-wedge[157], closed-wedge[158], and dome-shaped[159,160] osteotomy. HTO should be performed with one-stage ACL reconstruction simultaneously, postoperative rehabilitation after HTO combined with ACL reconstruction can be performed in the same manner as rehabilitation after HTO alone. Long-term outcome regarding one-stage HTO and ACL reconstruction suggested that it is an effective and safe procedure[161]. A systematic review of studies on one-stage HTO and ACL reconstruction reported that the percentages of patients who received opening-wedge and closed-wedge HTO were 57.4% and 42.6%, respectively, and the percentages of patients who received hamstring and BTB autograft for ACL reconstruction were 85.6% and 12.8%, respectively[162]. Although currently available data indicates high patient satisfaction and high rate of returning sport after combined with HTO and ACL reconstruction[163], further studies are needed to compare clinical outcomes between combined with HTO and ACL reconstruction and HTO alone.

FUTURE ACL RECONSTRUCTION

Biological augmentation of ACL repair and reconstruction

In most studies, ACL repair resulted in failure or unfavorable results. Recently, however, experimental and clinical studies on biological augmentation of mesenchymal stem cells, platelet-rich plasma (PRP), or other biologic agents with scaffold are being conducted to assess the effects of such biotherapies on ACL repair and reconstruction[164,165].

The four main components of tissue engineering such as cells, growth factors, scaffolds, and mechanical stimuli, are combined using various methods of bioaugmentation. They have been increasingly explored to improve outcomes after surgical treatment of ACL injury[164,166-169].

Scaffolds: Stem cell-based tissue regeneration combined with scaffolds represent a novel treatment for torn ligaments[170-172]. 3D scaffolds seeded with mesenchymal stem cells yielded excellent results in osteointegration enhancement between the tendon and bone tunnel in ACL reconstruction with a rabbit model[173]. PRP combined with a gelatin sponge to prolong PRP bioactivity promotes mesenchymal stem cell proliferation *in vitro*[174].

Cell sources: The main cell sources are mesenchymal stem cells and ACL fibroblasts [175]. Mesenchymal stem cells have higher proliferation and collagen production rates than ligament fibroblasts[176]. ACL-derived human-induced pluripotent stem cells might be a promising cell source for ligaments and related tissue engineering applications[177].

Growth factors: PRP is obtained by plasma separation. PRP contains platelets, blood proteins such as fibrin, and a mixture of growth factors such as platelet derived growth factor, insulin-like growth factor, vascular endothelial growth factor, and transforming growth factor-beta, which are involved in general healing processes.

PRP has been used to treat knee OA and to promote ligament healing. Recently, it has been used experimentally in ACL reconstruction to promote graft maturation and osteointegration[178]. However, no clinical efficacy data have been reported yet[179, 180].

Mechanical stimulation: Mechanical stimuli and dynamic loading are necessary for ligaments to enhance matrix synthesis and maintain their strength[181]. Electrospinning has been effective for cell proliferation and extracellular matrix production of scaffolds for ligament tissue engineering[182]. However, whether any mechanical stimulation is required to implant tissue-engineered ACL constructs is controversial [175].

In recent studies, bioenhanced ACL repair had similar results as ACL reconstruction. These biotherapies are expected to reduce postsurgical OA and to be improved in the future.

Computer-aided surgery

In other clinical departments, robotic surgery with the da Vinci™ Surgical System (Intuitive Surgical, Sunnyvale, CA, United States) has become more widespread, mainly in large-scale hospitals. In orthopedic surgery, computer-assisted navigation has come to be used for spine surgery[183-186], total hip arthroplasty, and total knee arthroplasty[187,188].

There are four main types of applications for navigation systems in ACL reconstruction[189,190]: (1) Technical assistance of tunnel placement for tibial or femoral tunnel drilling; (2) Kinematic evaluation to analyze the biomechanical behavior of the ACL and surrounding structures during reconstructive surgery[191]; (3) Comparison of the effectiveness of different surgical techniques for making laxity measurements[192]; and (4) Navigation to improve clinical outcomes and cost-effectiveness of ACL reconstruction.

3D fluoroscopy-based navigation system: It is essential to perform preoperative planning using 3D computed tomography (CT) images before operation. A reference frame is rigidly attached to the femur with two half-pins at the beginning of surgery. An intraoperative 3D image of the distal femur is obtained with the C-arm of the image intensifier, which is equipped with a wireless tracker. The image is reconstructed into a 3D image on the computer screen. A navigation computer helps the surgeon visualize the entire area for bone tunnel creation. However, this system requires fixation with two half-pins in the lateral femur, which necessitates an additional skin incision and more drill holes[193,194].

CT-based navigation without intraoperative fluoroscopy: This system uses a preoperatively generated 3D model from CT images or intraoperative 3D bone morphing with an optical tracking system. The optical tracking system captures reference markers that are rigidly attached to the patient and surgical tools. After fixing the tracking markers, approximately 20 Landmark points are collected on the surface of the bone with probes[195-197].

Anatomical reconstruction using the anteromedial technique is associated with more risks including: (1) A short femoral tunnel; (2) Posterior wall blowout; and (3) Iatrogenic damage to the cartilage of the medial femoral condyle due to the more horizontal direction of the femoral tunnel in the 3D plane[41]. Navigation systems with enhanced registration accuracy can reduce surgical failures such as short femoral

tunnels and posterior wall breakage of the distal femur[195,198].

Image free navigation system: This method does not require preoperative CT or intraoperative fluoroscopy. The transmitters for the femur and tibia are fixed with pins to register intra- and extra-articular landmarks intraoperatively. Next, the transmitter is attached to the tibial drill guide to determine the location of the tibial bone tunnel. The same maneuver is used for the femoral bone tunnel[199].

There is considerable variability in intra-articular landmark identification with image- free navigation. There is a potential risk of miscalculating tunnel positions[200].

Guided drilling of the tunnel leads to errors as small as 2.5 mm in the footprint and in the orientation of the intra-operative video for guiding the drilling of the tunnel with a set of contours which is reconstructed by touching the bone surface with an instrumented tool[201].

There are some studies on the use of computer-assisted navigation for bone tunnel positioning and evaluating joint instability in ACL reconstruction[202-204]. Clinical, radiological, and functional comparisons between computer-assisted and conventional ACL reconstruction have found increased accuracy in femoral tunnel placement with the use of navigation systems compared with traditional techniques alone[196,204-207]. Some studies reported that computer-assisted navigation improved the accuracy of tunnel positioning[208-210]. For inexperienced surgeons, navigation systems could be useful in ACL surgery to avoid malpositioning of bone tunnels[211,212]. However, another study showed that experienced surgeons could achieve more accurate tunnel positioning than computer-assisted positioning[211]. Consequently, computer-assisted navigation has not become common in clinical practice.

Although currently available navigation systems can enable more accurate femoral tunnel positioning and assist less experienced surgeons[201], they are not cost-efficient and require extra time for registration of operative positioning data[190,213]. Moreover, there is no difference in clinical outcomes between ACL reconstruction with and without computer-assisted navigation[214]. Therefore, a completely new system with high efficacy needs to be developed.

3D fluoroscopy-based navigation systems might be useful for confirming the native ACL footprint in remnant-preserving ACL reconstruction[215,216]. Several studies have described the use of navigation-assisted surgery to increase the possibility of achieving adequate tunnel position in revision ACL reconstruction[194,217].

Kinematic assessment of knee laxity among different ACL surgical procedures have been evaluated[218,219].

With the advancement of robotic surgery, remote surgical assistance will be available. At present, most ACL reconstructions are performed in urban hospitals by arthroscopic surgery specialists. It is not recommended that ACL reconstruction would be performed by a surgical team with no training in the procedure. However, if remote surgery assistance becomes available, and ACL reconstruction can be performed in rural areas where advanced medical care is unavailable, it will be helpful for residents in these areas. The da Vinci™ Surgical System has already been used for remote surgery assistance in some hospitals. In laparoscopic surgery assisted by the da Vinci™ Surgical System, the robot arms in a local hospital are remotely controlled by an advising surgeon in an operating room of an advanced medical facility. Such remote assistance enhances the skills of surgeons in local hospitals. In the future, robot-assisted ACL reconstruction surgery could also be achieved by remote instruction or remote control of robot by an arthroscopic surgery specialist. We hope that these technologies make advanced ACL surgery available to people living in countries and regions where advanced ACL treatment is unavailable.

CONCLUSION

There is no question that ACL reconstruction is necessary in order for patients with ACL injury to maintain a high level of activities, including sports, and that the number of patients receiving ACL reconstruction will increase around the world. However, ACL reconstruction has not provided satisfactory results in terms of the rate of returning to sports and prevention of OA progression. To enable many more orthopedic surgeons to achieve excellent ACL reconstruction outcomes with less invasive surgery, further studies aimed at improving surgical techniques are warranted. Further development of robotic surgery technologies for ACL reconstruction is also required.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 October 28; 9(5): 438-454

DOI: 10.13105/wjma.v9.i5.438

ISSN 2308-3840 (online)

REVIEW

Weight regain after bariatric surgery: Promoters and potential predictors

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Author contributions: Demerdash HM is in charge of all the works of this manuscript.

Conflict-of-interest statement: There is no conflict of interest.

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Manuscript source: Unsolicited manuscript

Specialty type: Endocrinology and metabolism

Country/Territory of origin: Egypt

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): 0

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Abstract

Obesity is globally viewed as chronic relapsing disease. Bariatric surgery offers the most efficient and durable weight loss approach. However, weight regain after surgery is a distressing issue as obesity can revert. Surgical procedures were originally designed to reduce food intake and catalyze weight loss, provided that its role is marginalized in long-term weight maintenance. Consequently, it is essential to establish a scientifically standardized applicable definitions for weight regain, which necessitates enhanced comprehension of the clinical situation, as well as have realistic expectations concerning weight loss. Moreover, several factors are proposed to influence weight regain as psychological, behavioral factors, hormonal, metabolic, anatomical lapses, as well as genetic predisposition. Recently, there is a growing evidence of utilization of scoring system to anticipate excess body weight loss, along with characterizing certain biomarkers that identify subjects at risk of suboptimal weight loss after surgery. Furthermore, personalized counseling is warranted to help select bariatric procedure, reinforce self-monitoring skills, motivate patient, encourage mindful eating practices, to avoid recidivism.

Key Words: Weight regain; Bariatric surgery; Hormones; Diet; Exercise; Genetic factors

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Core Tip: Obesity constitutes an enormous health struggle worldwide. Weight regain after bariatric surgery is a distressing issue that requires extensive study; various influencing factors as well as predicting biomarkers must be considered carefully before making decision for surgery, selecting bariatric procedure as well as close long term monitoring and support are essential.

Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 22, 2021
Peer-review started: April 22, 2021
First decision: May 12, 2021
Revised: June 7, 2021
Accepted: September 14, 2021
Article in press: September 14, 2021
Published online: October 28, 2021

P-Reviewer: DeSousa K
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Yu HG



Citation: Demerdash HM. Weight regain after bariatric surgery: Promoters and potential predictors. *World J Meta-Anal* 2021; 9(5): 438-454
URL: https://www.wjgnet.com/2308-3840/full/v9/i5/438.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i5.438

INTRODUCTION

Morbid obesity is the consequence of cumulative imbalances between energy intake and energy expenditure[1]. It is a serious chronic disease causing various comorbidities reducing affected persons' well-being and lifespan. Bariatric surgery is an option in morbidly obese subjects when lifestyle and non-surgical strategies evidenced incompetent. It produces superior reduction in body weight along with relief of associated comorbidities compared to nonsurgical interventions[2]. There are several reputable techniques adopted in management of morbid obesity worldwide. However, Laparoscopic Roux-en-Y gastric bypass (LRYGB) and laparoscopic sleeve gastrectomy (LSG) are two most common weight loss procedures due to their significant weight reduction and durability[3,4].

Following bariatric surgery, body contouring occurs when operated subject finally achieves the required body mass index (BMI) in a comprehensive and stable basis, exhibiting proper nutritional, psychological and clinical aspects[5]. Since during weight loss, those subjects may encounter nutritional depletion along with metabolic adaptation, which compromises healing demands and the whole response to surgical stress[6,7]. Consequently, expected weight loss after successful surgical procedure progresses through several anticipated phases[3] (Table 1). However, stability phase outcome is frequently humbled in considerable number of patients, with regain of 5-10 kg, after weight stabilization which is expected and considered normal[4]. Though, in long-term surveillance, there is growing perception concerning the possibility of weight regain after bariatric surgery[6]. Kraschnewski *et al*[8] stated that less than 20% of operated subjects who achieved expected weight loss through various surgical procedures within the first year, experienced variable extent of weight regain within five years.

IDENTIFICATION OF POSTOPERATIVE WEIGHT REGAIN

In order to determine weight regain, it is essential to comprehend the definition of weight stability. Considering that it is expected to observe minor fluctuations in body weight. Rubin defined weight stability as less than 2.5 kg variation in body weight per month over the preceding three months[9]. Given that this issue is subjective; Various literatures proposed a postoperative period of 12-18 mo till body weight becomes stable, with no more fluctuations through the succeeding 4-6 mo[10-12]. Accordingly, definition for weight regain requires careful description by determining patient characteristics, such as baseline BMI, age, gender, surgical procedure, general health perceptions, preoperative comorbidities[13].

Voorwinde *et al*[14] introduced six definitions for weight regain after bariatric surgery based on reports of various researches: (1) An increase of more than 10 kg from body weight denoting weight regain after achieving desired weight stability; (2) An increase of more than 25% excess weight loss (% EWL) after achieving weight stability phase; (3) An increase in BMI of 5 kg/m^2 after attaining steady BMI; (4) Weight regain to a BMI more than 35 kg/m^2 from baseline after long standing follow up *e.g.*, five years; (5) Any weight regain; and (6) An increase more than 15% of total body weight from baseline after long period follow up *e.g.*, five years[14]. This is determined by using following equation (total body weight at end of follow up period – total body weight at baseline/ total body weight at baseline × 100 > 15[13,15].

Percentage of EWL refers to reduction in body weight after bariatric surgery. In brief, a subject whose ideal body weight ought to be 70 kg but weighs 130 kg before bariatric procedure, implies 60 kg excess body weight. Appropriately, an optimum weight loss response is generally termed as 50% EWL[10,16]. Then long term postoperative expected weight loss is approximately two-thirds (2/3) of initial body weight (60 kg excess weight) or 40 kg[17]. The ideal weight is customarily determined on the basis of body mass index of 25 kg/m^2[13]. This is obtained from a formula where the square of subject's height in meters is multiplied by 25. For instance, if

Table 1 Phases of weight changes after bariatric surgery

Phase name	Phase description
Weight loss Phase	Initial prompt weight loss period during which convalescent patient could lose up to 25 kg in the first month, this period persists for up to six months[3]
Pre-stability Phase	Afterwards, weight loss slacks off for approximately one year[3]
Stability Phase	Ultimately, through the subsequent two years usually convalescent patients attain lowermost body weight, (having lost about 50% of the excess body weight) is maintained in the majority (70%-80%) of patients, irrespective of surgical procedure performed[2]

subject's height is 1.8 m: (1.8 × 1.8) × 25 then ideal body weight is 81 kg[13].

In pursuit for clinically applicable outcome indicators, some authors referred to percentage of total weight loss (%TWL) as the most accurate metric for calculating weight loss post-operative[10,12]. They claimed that %TWL is minimally affected by perplexing preoperative variables as initial BMI, age, comorbidities particularly diabetes. Therefore, %TWL is simple, easy to estimate and understand[12,18]. It is often utilized in appraising behavioral and pharmacotherapeutic literature[18].

%TWL is determine by formula: (Pre-operative body weight – Current body weight) / (Pre-operative body weight) × 100[12].

Some authors recommend that %TWL applied routinely to quantify weight loss and suggest that ≥ 20%TWL within 12 mo become the established benchmark to identify good responders to weight loss surgery along with reporting of remission or improvement of obesity comorbidities. As well as to track long-term weight regain after bariatric surgery[18,19]. However, Corcelles *et al*[20] concluded that use of single variable such as %TWL to delineate weight-loss outcomes can generate deceptive evaluation of patients results. Consequently, they recommended use of more than one definition for weight loss including change in BMI or BMI loss percent EWL, percent %TWL, percent excess BMI loss, and percent total BMI loss[20].

Weight changes in two most common bariatric procedures: LRYGB vs LSG

In terms of short-term outcome; RYGB yields 60%- 80% of excess body weight loss (EBWL) within the first year. It is generally recommended for morbid obesity with BMI over 40[18,19]. On the other hand, LSG yields weight loss at a slower, steadier rate, about 60% of excess body weight is lost within first 18 mo[21,22]. However, several studies revealed that both procedures are equally effective measures for short-term obesity management (< 36 mo) in super obese subjects[23,24].

Pertaining to long-term outcomes; five years postoperative follow up significant weight regain is sporadic for patients undergoing RYGB 2.5%, whereas patients undergoing LSG 14.6%[11]. This conclusion was supported by other studies[25,26]. It is worth mentioning that bariatric surgery was originally intended to downsize the stomach in an attempt to reduce food intake and prompt weight loss, but research studies approved that it has a minimal role in long-term weight loss maintenance[15,16,27].

DETERMINANTS OF WEIGHT REGAIN

The etiology of weight regain tends to be multifactorial including behavioral and dietary habits, mental health, anatomical changes, hormonal variations and genetic aspects[27,28]. Certainly, it results from a combination of components, which vary among individuals' overtime[29]. Moreover, anticipation of weight regain should be mandatory in consultation with all parties; patient, surgeon and nutritionist prior to surgery, as it may influence patient's ability to avoid weight regain[28]. Madan *et al*[30] have observed that only 10% of patients could recall being enlightened about the possibility of weight regain after surgery.

Dietary habits and behavioral practices

Negative eating habits and lack of dietary modifications are risks to long-term postoperative failure. Therefore, to obtain maximum benefit from surgery, patients should be encouraged to adhere to appropriate dietary habits[31]. Since, weight regain commonly occur as result of poor assortment of healthy diets rather than excessive intake[29]. Sawamoto *et al*[32] reported that persistence of poor preoperative eating

behavior after surgery was associated with 68.6% failure rate. Also, Felsenreich *et al* [33] found that unhealthy eating habit correlated with rate of 59% weight regain within 10 years after surgery. Nikiforova *et al*[34] obtained similar results, they proposed the explanation for weight regain include; sleeve or pouch dilation according to procedure performed, enhanced ghrelin levels, lifestyle behaviors and lack of follow-up support. Additionally, Mitchell *et al*[35] found a higher incidence of weight regain among subjects who consumed limited number of meals, less than 5 small frequent meals, and often skipped breakfast. Owing to a false belief that decrease in number of meals aids supplementary weight loss, whereas actually ingestion of numerous small meals per day augments diet-induced thermogenesis and stimulates smaller insulin secretion[32].

Healthy dietary deeds acknowledged among weight loss maintainers[36]: (1) Commitment to daily intake of breakfast as it assists stabilization of blood glucose level[31]; (2) Consumption of ample amounts of water, as optimal hydration prevent oxidative stress, while losing more than 2% of body water causes altered body temperature control, increased both physical and mental fatigue[36]; (3) Greater protein intake about 1.0-1.5 g/kg of ideal body weight per day, restriction of sugar less than 5 gm per serving and less than 30% of daily calories fat[30,37]; and (4) Ingestion of nutrients with low glycemic index and high dietary fibers contents such as fruits and vegetables prolong intestinal absorption[31]. Accordingly, Nutritional follow-up monitoring is consistently regarded as a vital element of medical management after bariatric surgery [38].

Mental health and psychiatric disorders

In this respect adverse eating behaviors could be a crucial factor; including emotional binge eating described as episodes of overeating even when not hungry, on exposure to emotional stimuli or stress to extent of causing gastrointestinal discomfort[31]. Similarly, Grazing is repetitive unplanned eating of minor quantities of food with loss of control over this feeding, associated with some psychological disturbance[39]. Also, distracted passive eating implies hyperphagia in presence of an element disturbing one's attention from the quantity of food ingested as chatting with a friend or watching television[27,40]. Moreover, some individuals may display cravings for fats and sweets, described as addictive personality or could be regarded as cortisol-mediated response to stress[41]. Sugar craving phenomenon may possibly be induced by central metabolic actions, as serotonin or dopamine imbalance, altered leptin levels [41]. Cognitive behavioral consideration proves more effective in curing maladaptive eating disorders than sole nutritional counselling without managing psychological part[42,43]. Authentically, psychological conditions as grazing could lead to weight regain postoperatively, regardless of surgery type in about 16%-39% of patients post-operatively[44-46]. This considerably high incidence necessitates accurate assessment, close monitoring and attention to guidelines for nutritional management postoperatively recommending eating slowly, chewing food thoroughly, and increasing the frequency of meals[42].

Physical activity and lifestyle

Physical inactivity is considered a valuable component criticized of having a role in weight regain along with as sleep deprivation (sleeping less than the recommended amount) and television watching more than two hours daily, all may be associated with reduced sensitivity to the internal satiety signals[36]. Similarly, Petit *et al* deduced that sleep deprivation augments mood changes, upsets glucose metabolism and appetite regulation[47]. They suggested that sleep replenishes glucose stores in neurons while the awake cycle depicts recurrent glycogen breakdown[47].

Corresponding to American College of Sports Medicine proposals for weight loss and prevention of weight regain for adults; entailed performance of 150 min per week of moderate or vigorous physical activity [moderate < 3-6 metabolic equivalents (METs), vigorous > 6 METs][48]. In view that experts assessed exercise performance in MET. Where one MET is expressed as the amount of energy spent to rest quietly, for average adult one calorie per every 2.2 pounds of body weight per hour[49]. This signifies that moderate activity is enough to burn 3 to 6 times as much energy per minute than lying quietly[50]. American Society for Metabolic and Bariatric Surgery encourages the increase in physical activity to a minimum of 30 min per day[48]. Notably, within the first year postoperative, physical activity contributes to enhanced oxygen consumption with prompt adaptability in heart rate and improved lipid profile[51]. While, Freire *et al*[52] depicted that it has a negligible effect on short-term weight loss, but assists long term weight loss maintenance. Some studies reported that

subjects engaged in moderate to vigorous physical activity achieved a greater % EBWL compared to others less physically active 2-5 years postoperative[53-55].

Anatomical modifications

RYGB bears a long-term failure rate of 20%-35%, particularly in super-obese population (BMI > 50 kg/m^2) due to gastro-gastric fistula or disrupted staple line[56]. Gastro-gastric fistula is a communication between pouch and stomach remnant, which permits food to cross duodenum reducing restrictive and malabsorptive potentials of RYGB, but is alleviated by surgical revision as biliopancreatic diversion or duodenal switch[57]. Even though wide gastrojejunostomy or pouch dilatation could be anticipated, if pouch is > 6 cm in length or > 5 cm in width[58,59]. Although, subjects who develop large pouches (> 50 mL) may comprise comparable weight loss to those with normal sized pouches[59]. However, this never omit contribution of large pouch to weight regain[57,58].

Consequently, Dayyeh *et al*[59] found that dilated gastrojejunal stoma after RYGB produces early gastric pouch emptying, they concluded that its' diameter was correlated with weight regain. While in LSG weight regain may result from physiologic dilation of stomach remnant in the long run, or failure of complete removal of the gastric fundus[60]. This entails reduction in post-prandial satiety, allowing increased volume of food consumed, to the extent that 59% of patients regained more than 20% of their lost body weight[60,61].

Hormonal/metabolic imbalance

Summary of gut hormone changes after surgery: A chief focus after performing bariatric surgery is time interval of satiety hormones signifying early sense of fullness with smaller meals based on their release site[62]. Additionally, there is an overall agreement that great improvement in glycemic control accomplished are likely to be associated with alterations in the secretion of gastrointestinal hormones including hunger (ghrelin), satiety [glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY)], and energy balance[63].

LSG reduces the size of the stomach; stimulus for gut hormones secretion specifically GLP-1 is obtained by proximal alimentary signals, *e.g.*, increased cholecystokinin derived by entrance of hydrochloric acid, amino acids, or fatty acids into duodenum[64,65]. These changes influence release of ghrelin, PYY, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) as well as other gut hormones[65,66]. On the other hand, RYGB approach, eliminates the stomach and proximal small intestine, creating a small pouch and direct connection with distal gut, consequential variations in gut hormones secretion results from abrupt exposure of intestinal epithelium to nutrients, with subsequent stimulation of L- cells[66]. This is coupled with significantly suppressed ghrelin, elevated GLP1, PYY levels, along with high pH of undigested chyme possibly contributing to diminished food intake and altered energy expenditure, leading to weight loss[66,67]. Also, exclusion of the upper segment of the intestine, where the GIP producing K-cells are present, would entail diminished levels of GIP, and is likely to constrain fat accumulation and intuitively supports long-term weight loss maintenance[68].

Hormonal disparity associated with weight regain

GLP-1: Acts to lower blood glucose by stimulating insulin release and inhibiting glucagon secretion[65]. GLP-1 is decomposed by ubiquitous dipeptidyl peptidase-IV (DPP-IV) enzyme[69]. Conversely, DPP-IV expression seems to be mediated by epigenetic influences as hyper- methylation of the DPP-IV promoter due to unhealthy lifestyle or genetic predisposition[69]. Also, deregulation of metalloproteinases coincident with fibrosis in different adipose tissue depots were found to induce insulin resistance in adipocytes and skeletal muscle cells[70]. Specifically, morbidly obese subjects coupled with insulin resistance undergoing bariatric surgery, may confront suboptimal response of GLP-1 associated with possibility of weight regain[71]. Those subjects achieve better response in terms of glycemic control and weight loss upon management with postoperative DPP-IV inhibitors[72].

Ghrelin/leptin ratio: A range of subjects may display a robust neuroendocrine-metabolic starvation feedback to initial weight loss that promotes metabolic energy preservation and favors weight regain after bariatric surgery[73]. Furthermore, Serum leptin is expected to decline by 50% after the first postoperative week, with an additional decrease during the first postoperative year[67]. Consequently, good responders revealed greater ghrelin to leptin ratio during fasting and post-prandial preoperative assessment contrasted with those who encountered weight regain[73].

On the other hand, operated patients who gradually regain weight, encountered resistance to the satiety-inducing effects of leptin[74]. The condition is characterized by elevated circulating leptin levels and decreased leptin sensitivity[75].

Growth hormone: Growth hormone (GH) is another sporadic factor principally regulated by two hypothalamic peptide hormones: GH-releasing hormone (GHRH) and somatostatin[76]. Though, further brain signaling pathways are participating in the control of GH secretion. Obese subjects may exhibit multiple endocrine defects that correspond to body composition variations, as increased visceral fat and decreased fat-free mass[77]. Theoretically, combination of various somatotropic axis alterations might be accountable for diverse scores of GH and insulin growth factor-1 (IGF-1) deficiency in obese subjects. Among the underlying neuroendocrine alterations of low plasma GH levels in obesity, associated with GHRH, somatostatin dysregulation[77]. Few studied recommended preoperative evaluations of GH/IGF-1[78]. They proposed that prevalence of persisting GH and/or IGF-1 levels below the normal range for six months after surgery was correlated with a significantly insufficient weight loss[77,78]. The GH/IGF-1 axis is assessed by evaluating serum IGF-1 Levels and the GH peak after stimulation by (GHRH + arginine hydrochloride)[78].

Reactive hypoglycemia: Establishes another hypothesis for weight regain as a consequence of dumping syndrome, mediated by hypersecretion of GIP and GLP-1, which may induce B cell expansion and insulin hypersecretion as long term consequences[79,80]. Besides this, Hypoglycemia ensues in 64%-82% of subjects within 5 years postoperative. The assumption for its occurrence involve refined pancreatic B cells function and mass, improved insulin sensitivity, diminished ghrelin level[80,81]. Since glucose is perceived as a chief appetite mediator, several attacks of hypoglycemia due to substantial insulin hypersecretion after meals, may trigger appetite[82]. Relatedly, direct effect of insulin on lipid accumulation since one of major function of insulin is inhibition of lipolysis[81,83]. Hence existing information reveals that attenuated postprandial gut hormone feedback /or reactive hypoglycaemia may show a correlation between eating disorders and hormonal imbalance[75,84].

Female menopause: Female menopause would apparently reduce effectiveness of bariatric-surgery outcome; given that estrogens mediate the effects of reproductive axis function on weight regulation[85]. Asarian *et al*[86] concluded that surgery seems more effective for pre-menopausal women (or post-menopausal women receiving hormone replacement therapy) than climacteric women with lower levels of estrogen. Because estrogens powerfully regulate the satiating effect of gut hormones as GLP-1 [76,85].

Genetic predisposition

Authentically, it is largely proposed that genetic and environmental aspects could modify the outcome of bariatric surgery, especially in the long run[87,88]. Exceedingly high baseline BMI > 50 kg/m^2 is be associated with concomitant fast postoperative weight loss, besides greater risk of postoperative weight regain[89,90].

Numerous genes were identified as obesity related genes, and mutations of any produces rare monogenic forms predispose to obesity; including leptin gene, leptin receptor, pro-opiomelanocortin (POMC), melanocortin 4 receptor, melanocortin 3 receptor, fat mass and obesity-associated (FTO), insulin induced gene 2, G protein-coupled receptor 24, corticotropin releasing hormone receptor and corticotropin releasing hormone receptor[91]. Remarkably, mutations in the zone of leptin/melanocortin pathway in the central nervous system being essential in regulation of energy homeostasis appears to result in enhanced appetite and reduced satiety, consequently early postoperative failure[89].

Accordingly, generation of genome-wide association studies (GWAS) provides a reasonably rich source of information to illustrate the molecular mechanisms connecting gene regulation, lifestyle and environmental factors in defining the risk of obesity[92,93]. Thus GWAS has improved comprehension of common genetic variants and collectively elucidated approximately 6% of the variation in adult BMI[93,94]. Correspondingly, the current hypothesis asserts that patients carrying none or few number of risk alleles of obesity show more efficient weight loss after bariatric surgery than carriers of multiple risk variants[95]. Genetic risk scores is computed by summarizing risk related variants across the genome, through gathering information from various predictive single nucleotide polymorphism (SNP)[91].

Unfortunately, in spite of intensive genetic research, the molecular mechanisms are barely clarified[91,95]. The epigenetic alterations preceding or post-translational

regulatory genes without changes in the nucleotide sequence, particularly methylation of DNA cytosine (C) represents an extremely stable modification[96]. Particularly epigenetic markers, in subjects who regained the weight loss induced by nutritional or surgical intervention displayed differential DNA methylation patterns in leukocytes or subcutaneous adipose tissue or compared to subjects successfully maintained their weight loss over a short or long periods[97].

Eventually, the methylation of genes involved in metabolic pathways exhibited changes after bariatric surgery. For example, weight regain is associated with hyper methylation of POMC and in turn, revealed higher melanocyte stimulating hormone-positive neurons[97]. On the other side, low methylation of neuropeptide Y gene is linked to hunger and satiety controlling peptides as ghrelin[98]. Furthermore, distinctive variants of FTO gene interrelate with dietary subscription, as high-protein regime benefits weight loss and improvement of body configuration in carriers of the FTO risk allele rs1558902, while carriers of FTO rs9939609 allele attained better weight loss response to low-fat hypocaloric diet[99].

Another epigenetic element is small non-coding RNAs (21-22 nucleotides), they are crucial for post-transcriptional regulation of gene expression. Single-stranded micro-RNA (miRNA) binds to a complementary target messenger RNA (mRNA) to disrupt translational processes[100]. Some studies reported short- and long-term miRNA profile changes after bariatric surgery in diverse tissues of both animal and humans that were associated with weight regain/or failure to achieve the desired body weight [101,102]. Accordingly, Obesity provoked changes in miRNA concentration within adipose tissues which promotes chemotaxis of macrophages and other immune cells towards the adipocytes. These miRNAs further impose chronic low grade inflammation, which sequentially may alter miRNA profile[103]. For example, miR-365 and miR-574, are proposed in adipose tissue hypertrophy through regulation of Early B cell factor 2 specific brown fat selective role involved in adipocyte differentiation *via* regulation of Peroxisome proliferator-activated receptor Gamma PPARγ, a ligand-activated transcription factor, involved in numerous cellular functions as lipid metabolism, glucose homeostasis and impediment of oxidative stress[104]. Therefore, they may represent prospective targets for non-surgical therapy of obesity and postulate novel biomarkers for predicting bariatric surgery outcomes[103].

PREDICTORS OF WEIGHT REGAIN

An essential measure during evaluation of patients with obesity prior to surgery; is identification of attributes or biomarkers that could deduce improvement of the metabolic profile of candidates that benefits from surgery on long term. Also, to categorize subjects more susceptibility to regain weight after surgery[32].

A greater weight reduction during early weight loss phase postoperative

Initial prosperous weight loss within the first few months postoperative anticipated long term weight loss at 4 and 8 years, examining long-term impression of intensive lifestyle intrusion on obesity associated comorbidities and mortality[35,105]. Also, Vogels *et al*[106] assumed that comparatively high baseline BMI and fat mass were correlated with concomitant long-term weight loss maintenance during two years follow up period (< 10% regain). Alvarado *et al*[107] deduced better response upon requesting morbidly obese subjects to lose around 4.5-9 kg of weight, in few weeks instantaneously prior to bariatric surgery, they attained improved postoperative weight loss. Similarly, some studies assessed preoperative weight loss of less than 5% or 5%-10% of total body weight was accompanied with superior outcome[108,109], However, other studies concluded that extremely high baseline BMI above 50 kg/m^2 was correlated with inferior postoperative weight loss (below 50% EWL)[110,111].

Baseline insulin sensitivity

Insulin regulates numerous metabolic processes within serval organ in body. Consequently, reduced sensitivity to insulin action is termed insulin resistance, *i.e.*, suboptimal response of various metabolic functions to normal insulin levels in blood [112]. It has a prime influence in predicting vulnerability to weight regain, and may be concluded through insulin resistance as defined by HOMA-IR[113]. Hence high baseline plasma insulin may be indicative of postoperative insulin resistance[112]. Similarly, Antuna-Puente *et al*[114] proposed that subjects with high plasma triglyceride concentrations were found to be insulin resistant. Kong *et al*[115] recommended applying a combination of biomarkers including elevated baseline fasting plasma

insulin and inflammatory biomarkers as IL-6 levels, and the number of HAM56$^+$ cells (macrophages) in subcutaneous white adipose tissue (WAT) to anticipate opposition to weight loss as well as susceptibility to weight regain with a prediction accuracy of 75%. Estimation of pancreatic β cell function (insulin secretion) and insulin sensitivity were calculated by using following formulas[116,117]:

(1) HOMA-IR = [Fasting insulin (uU/mL) × Fasting blood glucose (nmol/L)]/22.5.

(2) HOMA-β = [20 × fasting insulin (uU/mL)]/[Fasting blood glucose (mmol/L)-3.5].

(3) Quantitative insulin-sensitivity check index (QUICKI)= 1/[log fasting plasma insulin (uU/mL) + log fasting blood glucose(mg/dL)].

(4) Oral glucose tolerance test (OGTT): Fasting blood samples are drawn, then subject ingest 75 gm glucose load for OGTT. Glucose and insulin are measured in all blood samples obtained, usually at 0, 60, and 120 min or only 0 and 120 min during the OGTT.

(5) Matsuda index = 10000√ (FPI × FPG) × (x GPC × x IPC). Where FPI is fasting plasma insulin expressed as μU/mL, FPG is fasting plasma glucose expressed as mg/dL, x GPC is mean plasma glucose concentration after the load and x IPC is the mean insulin concentration after the load[117].

Expected normal ranges for above parameters: HOMA-IR normal range is less than 1.4, HOMA-β reference value is less than 81.7, while QUICKI index is less than 0.4 and MATSUDA index non diabetic range is less than 4.5[116,117].

Plasma concentrations of total cysteine

Plasma cysteine level is strongly connected with enlarged fat mass, probabilities of developing obesity and could be used as predictor of weight regain[118,119]. Cysteine is an essential sulfur-containing amino acid, that can form disulfide linkages, which simultaneously regulate protein structure and stability[120]. Circulating total cysteine (tCys) exists either as free cysteine homogeneous (cystine) or mixed (*e.g.*, homocysteine-cysteine) disulfides, and albumin-bound cysteine[121]. While cellular cysteine is the rate limiting precursor of intracellular antioxidant glutathione and prevail principally in reduced form[122]. Since plasma tCys is predominantly oxidized, high levels tCys, presume unfavorable outcomes, and is often related to obesity[120].

In addition, Cysteine enhances the activity of stearoyl-CoA desaturase-1 (SCD-1), which is a key enzyme for synthesis of monounsaturated fatty acids. SCD-1 introduces a single double bond at the Δ9, 10 of long-chain acyl-CoA substrates[123]. Its chief products are palmitoleic acid and oleic acid; the largely copious fatty acids in cholesterol esters, membrane phospholipids and triglycerides[123]. The activity of SCD-1 is also associated with the levels of sulfur containing amino acid, particularly cysteine. So SCD-1 suppression results in diminished fat deposits (regardless of food intake), enhanced insulin sensitivity and greater basal energy expenditure[123,124]. Accordingly, high plasma tCys observed to be positively linked to elevated total cholesterol, low density lipoprotein-cholesterol and triglycerides[125]. As it effectively inhibits hormone-sensitive lipase, and promotes adipocyte triglyceride and glucose uptake[125]. Hanvold *et al*[126] concluded that profound tCys elevation two years after RYGB was associated with weight regain.

Plasma adipokines

Some adipokines have displayed the ability to influence weight regain owing to their roles in energy expenditure and or food intake as adiponectin, retinol-binding protein 4 (RBP4), angiotensin converting enzyme (ACE) activity[127].

RBP4: RBP4 is transport protein for retinol (Vitamin A) secreted mainly by the liver and to a lesser extent by the adipose tissue. Munkhtulga *et al*[128] stated that the minor allele of regulatory RBP4 SNP was found to augment adipocyte RBP4 expression and was correlated with elevated BMI in Asian population. Wang *et al*[129] reported higher baseline RBP4 associated with compromised weight loss, they elucidated increased release of RBP4 in obese WAT owing to the presence of hypertrophic adipocytes during weight regain. In view that RBP4 carrying retinol is a precursor of ligands of retinoid X receptor, which activates peroxisome proliferator activated receptors modulating transcription of genes involved in fat metabolism and adipogenesis[130]. In this respect, elevated RBP4 level reflects stimulated adipocyte proliferation, adipogenesis and accordingly weight regain[131].

Additionally, RBP4 Links to insulin resistance through RBP4 associated effects comprising increased hepatic gluconeogenesis by accentuating the exhibition of phosphoenolpyruvate carboxykinase enzyme in the liver which simultaneously

suppresses insulin signaling in skeletal muscle by blocking insulin-stimulated phosphorylation of insulin receptor substrate-1[132]. Moreover, glucose transporter GLUT4 protein level in human adipocytes correlates positively with the rate of glucose clearance and inversely with circulating RBP4 level[133].

ACE: ACE gene polymorphisms are associated with BMI and obesity[134]. Provided that immense amounts angiotensin II (Ang II), product of ACE activates adipocyte differentiation and consequently influence adipose tissue mass[135]. Moreover, Wang *et al*[136] observed that greater reduction in ACE level was coupled with stable body weight during follow-up and abstained tendency to weight regain. Velkoska *et al*[137] demonstrated the effect of ACE inhibitors on body weight management by reducing body water or *via* reduction in energy intake.

ACE is a carboxypeptidase that is expressed in many tissues including adipose tissue but predominantly in the vascular endothelium, brain and lung. The enzyme is anchored to cell membranes and is shed into the plasma by enzymatic cleavage[138]. Furthermore, a positive association between the weight loss induced change in serum ACE concentration and weight regain during *ad libitum* feeding (free feeding)[138, 139]. A causal association appears between ACE and weight regain. However, the underlying mechanism remains unclear, in spite of the fact that ACE plays a role in many processes other than the regulation of blood pressure, such as inflammation, fibrosis and the regulation of food intake by the hypothalamus by reinforcing thermogenesis (though corticotrophin releasing hormone-autonomous mechanism)[136].

Fibroblast growth factor 21: Fibroblast growth factor 21 (FGF21) is a "myokine." that stimulates the oxidation of fatty acids, production of ketone bodies, and inhibition of lipogenesis[141]. Kharitonenkov *et al*[141] proposed that it could be a prospective metabolic regulator and a potential anti-diabetic drug. FGF21 mRNA is expressed in gastrointestinal tract, brain, skeletal muscle, brown adipose tissue, and heart[142]. FGF21 is a molecule with a very short half-life of 1-2 h in absence of specific stimuli, due to enzymatic cleavage by fibroblast activation protein α (FAP), a serine protease that inactivates FGF21[143]. Interestingly, FAP is secreted from muscle during physical exercise[144]. Also, FGF21 is removed from circulation by renal clearance[140].

FGF21 acts as a fasting-induced hormone intended for the adaptive response to starvation and consumption of energy derived from tissues breakdown[145]. Several studies hypothesized relationship between energy expenditure, body weight regulation and FGF21[145-147]. Through provoking energy expenditure with acute low-protein diet 3% causes almost an average energy expenditure (adaptive thermogenesis) can identify individuals who are able to disperse the excess calories consumed as heat ("spendthrift") opposed to those who are incapable ("thrifty")[145]. The extent of the elevation in serum FGF21 level in condition of acute low-protein overfeeding is positively correlated with the acute change in 24-h energy expenditure. While poor responders (Thrifty) show an attenuated FGF21 response to acute low-protein feeding, therefore are vulnerable to weight gain over 6 mo[145,146]. Provided that individuals don't suffer liver, cardiac, kidney or muscle disease which may interfere with test results[147].

CONCLUSION

Bariatric surgery is a tool to achieve significant weight loss, to obtain maximum benefit must adapt pre and postoperative follow up. Also, the role of a nutritionist in bariatric surgery team has expanded ahead diet counseling, to address individual barriers and counsel morbidly obese subjects by ensuring they understand selected bariatric procedures, offering education, identify individual factors that may predict weight regain, reinforce self-monitoring skills, encourage mindful eating practices, supply appropriate nutritional supplements, and motivate daily physical activity, provide close support and follow-up to avoid relapse.

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World Journal of Meta-Analysis

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World J Meta-Anal 2021 October 28; 9(5): 455-461

DOI: 10.13105/wjma.v9.i5.455

ISSN 2308-3840 (online)

MINIREVIEWS

Review of the effects of SARS-CoV2 infection and COVID-19 on common pediatric psychiatric illnesses

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Author contributions: Balaram K, Ahmed M, and Marwaha R contributed equally to this work; Balaram K and Ahmed M designed the search criteria and protocol and wrote the manuscript; Marwaha R supervised the search and writing processes and provided final edits; All authors have read and approve the final manuscript.

Conflict-of-interest statement: The authors of this paper declare no conflicts of interest.

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Manuscript source: Invited manuscript

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Abstract

Severe acute respiratory syndrome coronavirus-2 is a novel coronavirus strain that causes pneumonia and acute respiratory distress syndrome along with other morbidities, collectively known as coronavirus disease 2019 (COVID-19) disease. There has been widespread discussion about the psychological impact of COVID-19 particularly on children and adolescents. There have been overarching negative effects with regards to decreased physical activity, more screen time, increasingly unhealthy diets, and irregularities in sleep/wake schedules. This, coupled with disruptions in ongoing mental health treatment and associated support structures, has caused unprecedented declines in the emotional and psychosocial wellbeing of children and adolescents. This review aims to systematically review the literature to provide a general overview of the ways in which COVID-19 has affected common psychiatric illnesses in children and adolescents. The included articles in all subsections concluded that symptoms of these common childhood psychiatric disorders have generally been exacerbated by the COVID-19 pandemic. This review indicates that quarantine and the consequent isolation have had multiple significant and consistent negative implications on the mental health of children and adolescents. Our study indicates that there should be increased vigilance among providers and families to mitigate the negative psychological effects that the COVID-19 pandemic has on children with common childhood psychiatric disorders.

Key Words: COVID-19; Depression; Anxiety; Attention deficit hyperactivity disorder; Obsessive-compulsive disorder; Tourette's; Children; Adolescents

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Core Tip: The included articles in all subsections concluded that symptoms of these

Specialty type: Psychiatry

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: March 30, 2021
Peer-review started: March 30, 2021
First decision: July 30, 2021
Revised: August 14, 2021
Accepted: October 20, 2021
Article in press: October 20, 2021
Published online: October 28, 2021

P-Reviewer: Bianchi F
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

common childhood psychiatric disorders have generally been exacerbated by the coronavirus disease 2019 (COVID-19) pandemic. Our study indicates that there should be increased vigilance among pediatricians and families to mitigate the negative psychological effects that the COVID-19 pandemic has on children with common childhood psychiatric disorders. This calls out for pediatricians, psychiatrists, and all providers alike to remain cognizant of these effects and work collaboratively towards measures to reduce the psychological impact of COVID-19.

Citation: Balaram K, Ahmed M, Marwaha R. Review of the effects of SARS-CoV2 infection and COVID-19 on common pediatric psychiatric illnesses. *World J Meta-Anal* 2021; 9(5): 455-461
URL: https://www.wjgnet.com/2308-3840/full/v9/i5/455.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i5.455

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV2), is a novel coronavirus strain that causes pneumonia and acute respiratory distress syndrome along with other morbidities, collectively known as coronavirus disease 2019 (COVID-19) disease [1]. The World Health Organization declared SARS-CoV2 infection to be a global pandemic in March 2020. As of February 2021, there are upwards of 110 million cases of SARS-CoV2 infection worldwide with over 28 million cases and 500000 total deaths documented in the United States. In addition to significant medical impact, there has also been a profound psychologic impact because of the pandemic, particularly among vulnerable populations. There has been widespread discussion about the psychological impact of COVID-19 particularly on children and adolescents[2].

Since the start of the pandemic, the transition to online-based education, increasing school closures, and the alteration of normal adolescent social activities have caused an unmitigated disruption in the lives of children[2]. In general, the greatest impacts appear to be losses in areas of daily routine, institutional support structures, and social connection[2]. Though these effects are generally attributed to the cessation of in-person learning in schools, it is generally assumed that there have been overarching detrimental effects with regards to decreased physical activity, more TV and screen time, increasingly unhealthy diets, and irregularities in sleep/wake schedules[2].

This, coupled with disruptions in ongoing mental health treatment and associated support structures, has caused unprecedented declines in the emotional and psychosocial wellbeing of children and adolescents[2].

This review aims to systematically review the literature to provide a general overview of the ways in which COVID-19 has affected common psychiatric illnesses in children and adolescents.

For this review, we performed multiple literature searches of PubMed, Cochrane and PsycInfo to find articles (Figures 1-5). The first search used the keywords "COVID 19," "children," and "OCD," the second search used the keywords "COVID 19," "children," and "ADHD," the third search used the keywords "COVID 19," "children," and "Tourette's syndrome," the fourth search used the keywords "COVID 19," "children," and "depression," and the fifth search used the keywords "COVID 19," "children," and "anxiety." The search was not initially restricted by study design or language but was limited to articles published between January 1 to December 31, 2020. Review articles published in or translated to English were included. Full-text references cited in these articles were also researched for additional relevant studies. The inclusion criteria were on-topic articles discussing the effect of COVID-19 on common pediatric psychiatric disorders. The exclusion criteria were any study designs that were case reports, surveys, or study protocols, or articles that were considered to be off topic.

OBSESSIVE COMPULSIVE DISORDER

A total of 12 articles were identified through a literature search. There were no clinical

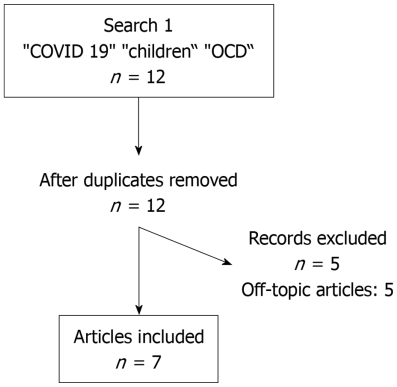


Figure 1 PRISMA table for Search 1.

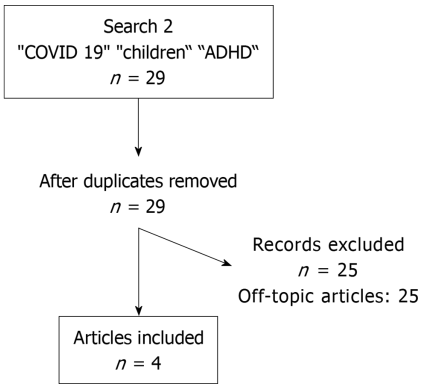


Figure 2 PRISMA table for Search 2.

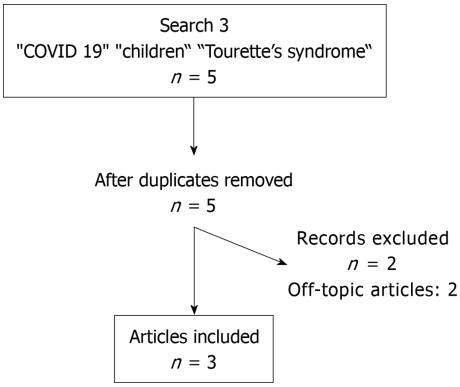


Figure 3 PRISMA table for Search 3.

trials, randomized controlled trials or meta-analysis that were identified. After excluding articles that were off-topic, seven articles remained for inclusion (Figure 1).

Though the mean age of onset for those with obsessive-compulsive disorder (OCD) is 19.5 years, childhood onset does occur and generally persists throughout adulthood [3]. According to the diagnostic and statistical manual of mental disorders, 5th edition, 25% of males have onset of symptoms before age 10. Characterized by obsessions and compulsions, OCD can present with a varying combination of symptoms including intrusive and persistent unwanted thoughts, repetitive checking, fears of contamination, feelings of worry and disgust, and repetitive behaviors or patterns of behaviors that an individual is compelled to perform[4,5]. In general, symptoms of OCD are exacerbated by acute stressors, such as trauma.

Due to the COVID-19 pandemic, children and adolescents who are susceptible to OCD are most likely to be affected[6]. There were several studies that concluded that children and adolescents diagnosed with OCD experienced a considerable worsening

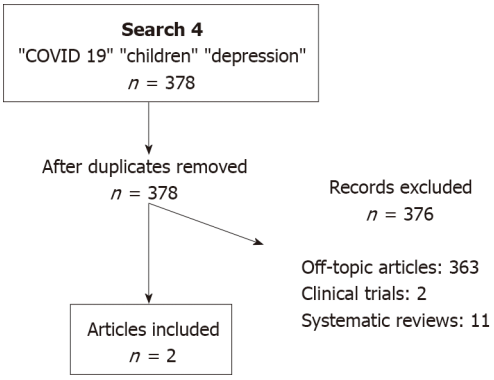


Figure 4 PRISMA table for Search 4.

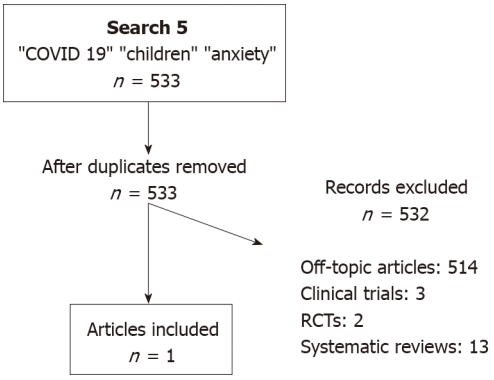


Figure 5 PRISMA table for Search 5.

of their symptoms[6-10]. One particular study concluded that this significant worsening was most prevalent in those with an earlier age of symptom onset and in those with a family history of attention deficit hyperactivity disorder (ADHD)[7]. Another study also identified a substantial increase in contamination fears and cleaning or washing obsessions due to general fears of exposure to infection brought on by the pandemic[8,9]. Contrastingly, there has also been widespread advocacy of infection control measures, such as frequent handwashing, and dissemination of information on avoiding exposure/contamination. These measures may actually lead to those suffering from severe OCD symptoms to feel less stigmatization over their cleansing obsessions[9,10]. They may instead feel more acceptance over their symptoms, as their fears become well-founded and "real" and their ritualized behaviors became generally widespread and even encouraged[9,10]. Therapeutic interventions, such as cognitive behavioral therapy, are designed to achieve symptomatic relief by targeting behaviors like excessive hand washing and intrusive thoughts of contamination. This can lead to a cognitive dissonance in those with OCD as the COVID-19 pandemic has led to a widespread and public campaign to encourage these behaviors.

ADHD

A total of 29 articles were identified through a literature search. There were no clinical trials, randomized controlled trials or meta-analysis that were identified. After excluding articles that were off-topic, four articles remained for inclusion (Figure 2).

ADHD, characterized by persistent patterns of inattention and/or hyperactivity/impulsivity that interferes with functioning, is one of the most prevalent psychiatric disorders of childhood[3,11]. In general, children diagnosed with ADHD have been noted to experience an exacerbation of symptoms throughout the COVID-19 pandemic[11-14]. In addition, children with the hyperactive phenotype of ADHD may find the constraints of quarantine and lockdown to be particularly difficult to accommodate[15]. Several studies noted an increase in symptoms such as

hyperactivity, irritability, emotional outbursts, and defiant or disruptive behavior[12-14]. There was also a noted increase in problematic behaviors from caregivers, such as irritability or aggressive behavior directed towards the child. These changes were generally attributed to both the nonspecific emotional stress caused by the pandemic and the lack of daily structure and increasingly flexible school/Learning schedules [13].

TOURETTE'S DISORDER

A total of five articles were identified through a literature search. There were no clinical trials, randomized controlled trials or meta-analysis that were identified. After excluding articles that were off-topic, three articles remained for inclusion (Figure 3).

Public health measures aimed at controlling the spread of the COVID-19 pandemic have led to some changes in the symptomatic presentation of Tourette's disorder[15-17]. The advent of online learning and social distancing measures have decreased the social burden of tics, and resulting psychological distress, felt by children in school and other learning environments[16]. In contrast, several other non-obscene socially inappropriate tics - such as spitting, coughing, sneezing, or inability to maintain interpersonal distance with touching – have become more socially unacceptable[15]. This can lead to increased anxiety and distress in children with these symptoms who may feel undue pressure to modify or control these behaviors[15]. This increased anxiety can lead to a secondary increase in tic symptoms[15]. In general, children with Tourette's disorder were observed to have overall clinical decline with either a worsening of existing symptoms or a resurgence of previously-controlled symptoms [15-17].

DEPRESSION AND ANXIETY

A total of 378 articles were identified through a literature search by using the keywords "COVID," "depression," and "children." There were two clinical trials, two meta-analysis, and eleven systematic reviews. There were no randomized controlled trials that were identified. The clinical trials were excluded as they were off topic. The systematic reviews were excluded due to the overlap from within the meta-analyses. After excluding articles that were off-topic, two meta-analyses remained for inclusion (Figure 4).

A total of 533 articles were identified through a literature search by using the keywords "COVID," "anxiety," and "children." There were three clinical trials, four meta-analysis, two randomized controlled trials, and thirteen systematic reviews. The clinical trials were excluded as they were off topic. The randomized controlled trials were excluded as they were not pertinent to the population subgroup of our review and dealt with the ailments in the adult population. The systematic reviews were excluded due to the overlap from within the meta-analyses. After excluding articles that were off-topic, one meta-analysis remained for inclusion (Figure 5).

Both anxiety and depression are major ailments amongst the youth that are often under-reported and under-recognized. Data by SAMHSA, or the Substance Abuse and Mental Health Services Administration, shows that the overall prevalence of depression for adolescents aged 12 to 18 is 13.1% as opposed to 7.1% for adults. Similarly, the rates are 31.9% and 19.1% when it comes to anxiety amongst adolescents, and adults respectively. This means that the relative prevalence of these disorders already remains higher amongst the adolescents as compared to adults.

COVID-19 and its consequent effects have further negatively impacted the mental health of children and adolescents. Multiple studies have shown that social isolation and the resultant loneliness were associated with increased risk of both depression and anxiety[18-25]. Anxiety was seen to be more prevalent amongst children maintaining quarantine, and this was thought to be likely mostly due to loss of primary caregiver's job, financial instability leading to a dearth in provision of basic amenities[22]. It was also noted that the time period of isolation was more important for symptomatology of mental health ailments than the severity of isolation[23].

The studies noted certain interesting factors. There was seen to be a mild difference between the sexes as well, with loneliness being more strongly associated with social anxiety in boys and depression in girls[23]. Certain primary school students who viewed COVID-19 as a serious disease had increased anxiety and somatic symptoms [2]. One study mentioned that children who practiced social distancing for fear of

getting infected were predisposed to anxiety disorders. The same study also mentioned that children who engaged in social distancing secondary to peer pressure had a higher likelihood of depression[24]. This illustrates a thought-provoking difference in symptoms based upon the motivator for social distancing in the first place. Interestingly an age-related difference was also discernible in certain study populations. High school children reported higher number and severity of symptoms of anxiety and depression as compared to their primary and middle school counterparts[24,25]. Certain salient causes of increased depression levels in the study population were also noticed such as internet/smartphone addiction and affected friends/family members in the past.

On the contrary a few of the factors protective towards depression were lesser time spent on the internet, adequate coping mechanisms, increased exercise time, and having a sibling[19,20]. It should come as no surprise that the pandemic meant more children/adolescents, and adults alike ended up spending increased time on the internet and lesser time outdoors. Similarly, children and adolescents with worry and fear about COVID-19 had higher rates of depression, whereas remaining optimistic amidst these times had the opposite effect[2].

CONCLUSION

The included articles in all subsections concluded that symptoms of these common childhood psychiatric disorders have generally been exacerbated by the COVID-19 pandemic. This was primarily attributed to the restricting quarantining regulations and the public fear of SARS-CoV2 infection. For instance, the routine learning schedule implemented at public schools that had helped to provide structure to children with ADHD have been lost while quarantining at home. This in turn led to worsening ADHD symptoms and behaviors. The public fear of COVID-19 and the widespread encouragement of frequent handwashing have both exacerbated contamination fears in those with OCD experience and worsened repetitive sanitizing behaviors. While children with Tourette's Disorder may have experienced less psychosocial distress over their tics by being away from school, they may still face difficulties with controlling symptoms because of increased anxiety over their tic behaviors breaking social-distancing rules.

This review portends that quarantine and the consequent isolation have had multiple significant and consistent negative implications on the mental health of children and adolescents. The stigmata of these consequences can be detected even months later beyond quarantine[25]. In general, a lack of vigilance and treatment for pandemic-associated effects on childhood psychiatric disorders may propagate significantly negative and refractory long-term sequelae into adulthood. Our study indicates that there should be increased vigilance among clinicians and families to mitigate the negative psychological effects that the COVID-19 pandemic has on children with common childhood psychiatric disorders. This calls for pediatricians, psychiatrists, and all healthcare providers alike to remain cognizant of these effects and work collaboratively towards measures to reduce the psychological impact of this already menacing physical ailment.

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World J Meta-Anal 2021 October 28; 9(5): 462-473

DOI: 10.13105/wjma.v9.i5.462

ISSN 2308-3840 (online)

META-ANALYSIS

Maturation of robotic liver resection during the last decade: A systematic review and meta-analysis

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Supported by Grants-in-Aid from JSPS KAKENHI, No. JP 20K10404 (to Mizuguchi T); the Hokkaido

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Abstract

BACKGROUND

Minimally invasive hepatectomy techniques have developed rapidly since 2000.

Hepatitis B Litigation Orange Fund, No. 2059198; Terumo Life Science Foundation, No. 2000666; Pfizer Health Research Foundation, No. 2000777; the Viral Hepatitis Research Foundation of Japan, No. 2000638; Daiichi Sankyo Company, No. 2109540; Shionogi and Co., No. 2109493; MSD, No. 2099412; Takeda, No. 2000555; Sapporo Doto Hospital, No. 2039118; Noguchi Hospital, No. 2029083; Doki-kai Tomakomai Hospital, No. 2059203; and Tsuchida Hospital, No. 2069231.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist statement, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist statement.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: April 13, 2021
Peer-review started: April 13, 2021

Pure laparoscopic liver resection (LLR) has become the primary approach for managing liver tumors and procuring donor organs for liver transplantation. Robotic liver resection (RLR) has emerged during the last decade. The technical status of RLR seems to be improving.

AIM

To conduct a systematic review and meta-analysis comparing the short-term clinical outcomes of LLR and RLR over two 5-year periods.

METHODS

A systematic literature search was performed using PubMed and Medline, including the Cochrane Library. The following inclusion criteria were set for the meta-analysis: (1) Studies comparing LLR *vs* RLR; and (2) Studies that described clinical outcomes, such as the operative time, intraoperative bleeding, intraoperative conversion rate, and postoperative complications.

RESULTS

A total of 25 articles were included in this meta-analysis after 40 articles had been subjected to full-text evaluations. The studies were divided into early (n = 14) and recent (n = 11) groups. In the recent group, the operative time did not differ significantly between LLR and RLR (P = 0.70), whereas in the early group the operative time of LLR was significantly shorter than that of RLR (P < 0.001).

CONCLUSION

The initial disadvantages of RLR, such as its long operation time, have been overcome during the last 5 years. The other clinical outcomes of RLR are comparable to those of LLR. The cost and quality-of-life outcomes of RLR should be evaluated in future studies to promote its routine clinical use.

Key Words: Hepatectomy; Laparoscopy; Robot; Operation time

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Core Tip: A systematic review and meta-analysis comparing the clinical outcomes of laparoscopic liver resection (LLR) and robotic liver resection (RLR) was conducted. A total of 25 studies were included in the meta-analysis. In the recent studies, operative time did not differ significantly between LLR and RLR (P = 0.70), whereas in the early studies LLR was associated with significantly shorter operative times than RLR (P < 0.001). The initial disadvantages of RLR have been overcome during the last 5 years.

Citation: Ishinuki T, Ota S, Harada K, Meguro M, Kawamoto M, Kutomi G, Tatsumi H, Harada K, Miyanishi K, Takemasa I, Ohyanagi T, Hui TT, Mizuguchi T. Maturation of robotic liver resection during the last decade: A systematic review and meta-analysis. *World J Meta-Anal* 2021; 9(5): 462-473
URL: https://www.wjgnet.com/2308-3840/full/v9/i5/462.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i5.462

INTRODUCTION

Surgery is a curative treatment for liver tumors[1]. The development of surgical devices has promoted minimally invasive surgery (MIS), including minimally invasive liver resection[2]. Therefore, the concept of 'big surgeons, big incision' has become a myth[3]. Minimal skin wounds are preferable, and patients who undergo laparoscopic liver resection (LLR) recover faster without somatic pain than those that undergo open liver resection[1,4].

MIS has significant clinical benefits, *e.g.*, it results in faster recovery, less pain, and shorter hospital stays[5]. On the other hand, long operation times and the associated higher costs were reported as disadvantages of the MIS approach[4,5]. However, the disadvantages of the MIS approach might be ameliorated as surgeons gain experience

First decision: June 24, 2021
Revised: July 1, 2021
Accepted: August 23, 2021
Article in press: August 23, 2021
Published online: October 28, 2021

P-Reviewer: Wang K
S-Editor: Liu M
L-Editor: Filipodia
P-Editor: Yu HG



[6]. Initially, LLR was reported to have various clinical benefits but result in longer operation times[4].

Robotic surgery has gained popularity since 2000[7]. Although robotic towers occupy space in the operating room, the skill of surgeons can be enhanced by robotic technology, such as "wristed instruments", "tremor cancellation", "enhanced dexterity", and "3D vision"[8,9]. These technologies are considered to reduce 93% of errors associated with human skill[8].

Total robotic liver resection (RLR) is limited to minor liver resection, which does not require the liver to be mobilized[10]. Furthermore, the robotic approach is only used for parenchymal dissection during laparoscopic surgery[11]. Therefore, the clinical outcomes of LLR and RLR should be similar[5,12]. We systematically reviewed the literature in which the clinical outcomes of LLR and RLR were compared. We divided the studies according to the year of publication to determine how the clinical outcomes of these techniques have changed over time. Early studies were defined as those published in 2016 or earlier. Recent studies were defined as those published in 2017 or later. We also examined the current status of RLR through a meta-analysis.

MATERIALS AND METHODS

Literature search

The Preferred Reporting Items for Systematic Reviews And Meta-Analyses (PRISMA) statement guidelines were followed when obtaining and reporting the meta-analysis data[13]. The PICOS scheme was employed when reporting the inclusion criteria. A systematic literature search of PubMed and MEDLINE, including the Cochrane Library, was performed independently by two authors (Ishinuki T and Ota S). The search was limited to human studies whose findings were reported in English. No restriction was set with regard to the type of publication, the publication date, or publication status. Patients of any age or sex who underwent liver resection for any hepatic lesion were considered, as outlined in the PICOS scheme. The search strategy was based on different combinations of words for each database. For the PubMed database the following combination was used: ("hepatectomy"[MeSH Terms] OR "hepatectomy"[All Fields] OR ("liver"[All Fields] AND "resection"[All Fields]) OR "liver resection"[All Fields]) AND ("laparoscopie"[All Fields] OR "laparoscopy"[MeSH Terms] OR "laparoscopy"[All Fields] OR "laparoscopies"[All Fields]) AND ("robot"[All Fields] OR "robot s"[All Fields] OR "robotically"[All Fields] OR "robotics"[MeSH Terms] OR "robotics"[All Fields] OR "robotic"[All Fields] OR "robotization"[All Fields] OR "robotized"[All Fields] OR "robots"[All Fields]). For the MEDLINE database, including the Cochrane Library database, the following combination was used: #1. liver.mp. [mp=title, abstract, full text, caption text], #2. resection.mp. [mp=title, abstract, full text, caption text], #3. robot.mp. [mp=title, abstract, full text, caption text], #4. 1 and 2 and 3.

Selecting policy of the studies

The independent authors have read the primary studies searched in the database. Similar studies and unrelated studies were excluded. The inclusion criteria for the statistical analysis were following: (1) Studies comparing LLR and RLR; (2) Studies reporting at least one clinical result or variable; and (3) If any institution reported multiple studies, only the recent and the excellent study was selected. The policies of the exclusion were following: (1) The studies dealing with liver transplantation; (2) Reviews, opinions, comments, letters, and case reports; and (3) The studies were impossible to reproduce. The Cohen kappa statistic was used to quantify assess the agreement among the researchers.

PROSPERO was used for the protocol registration (#CRD42021234405).

Data extraction

The independent authors extracted the following initial data: (1) The name of authors, year, and quality of study; (2) The etiology of the disease; and (3) The period of the evaluations.

Bias assessment

The publication bias was assessed by the Newcastle-Ottawa Scale: NOS (http://www.ohri.ca/), as they included observational studies. The NOS consists of domains for the patient selection, comparability of study groups, and outcome

Table 1 Frequency of each type of liver resection in the studies published in 2016 or earlier

Ref.		Laparoscopic liver resection					Robotic liver resection				
		Seg	LLS	LH	RH	EH	Seg	LLS	LH	RH	EH
Berber *et al*[17], 2010	Case	12	11				6	3			
Ji *et al*[18], 2011	Case	9	7	3	1		1	4	6	2	1
Lai *et al*[19], 2011	Cohort	6	4				6	3			
Lai *et al*[20], 2012	Cohort	9	8				12	17	2	1	
Packiam *et al*[21], 2012	Case		18					11			
Troisi *et al*[9], 2013	Case	149	39	16	17	2	38	2			
Spampinato *et al*[22], 2014	Case			9	15	1			8	16	1
Tranchart *et al*[23], 2014	Case	22	5	1			22	5	1		
Tsung *et al*[12], 2014	Case	72		21			36		21		
Wu *et al*[24], 2014	Case	28	31	8	2		8	24	7	12	1
Yu *et al*[25], 2014	Case		6	11				10	3		
Croner *et al*[26], 2016	Case	ND					ND				
Kim *et al*[27], 2016	Case		31					12			
Lai *et al*[28], 2016	Cohort	25	9		1		45	29	6	20	1
Lee *et al*[29], 2016	Case	34	30	2			17	39	10	4	
Montalti *et al*[30], 2016	Case	72					36				

EH: Extended hemi-hepatectomy; LH: Left hepatectomy; LLS: Left lateral segmentectomy; ND: Not properly described; RH: Right hepatectomy; Seg: Segmentectomy.

assessment. The low risk of bias results in a score of 9 points. We considered studies that scored ≥ 7, 4-6, and < 4 to be high, moderate, and low quality, respectively[14].

Statistical analyses

RevMan software (version 5.3.; The Cochrane Collaboration) was used for the meta-analysis. For continuous variables, the differences between groups were compared using the inverse-variance method. On the other hand, dichotomous outcomes were compared using the Mantel-Haenszel method. The Egger's test for publication bias was performed using EZR (version 1.54; https://www.softpedia.com/get/Science-CAD/EZR.shtml)[15].

The χ^2 test was used to evaluate heterogeneity, and the Cochran Q and I^2 statistics were reported. The I^2 value describes the percentage variation between studies in degrees of freedom. Low, moderate, and high heterogeneity were defined based on cut-off values of 25%, 50%, and 75%, respectively[16].

All results were considered significant at P values of < 0.05.

RESULTS

The PRISMA flow diagram for this study is shown in Figure 1. The database search for relevant studies resulted in 1,068 studies being identified. We excluded 922 studies because of duplication, and the titles and abstracts of the remaining 148 studies were screened. As a result, we reviewed 40 full-text articles to evaluate their eligibility further. We excluded 8 studies for which the outcome involved a non-target comparison, and 4 studies for which the data were not available. Finally, we included 28 studies in our meta-analysis.

The data regarding the frequency of each type of liver resection in the selected studies are shown in Tables 1 and 2. Table 1 shows the data for the studies published in 2016 or earlier[9,12,17-30]. Table 2 shows the data for the studies published in 2017 or later[31-42]. No randomized controlled trials (RCT) comparing the clinical outcomes of LLR and RLR were identified. All of the selected publications related to observa-

Table 2 Frequency of each type of liver resection in the studies published in 2017 or later

Ref.		Laparoscopic liver resection					Robotic liver resection				
		Seg	LLS	LH	RH	EH	Seg	LLS	LH	RH	EH
Efanovet al[31], 2017	Cohort	ND					ND				
Magistriet al[32], 2017	Case	24					14	6		2	
Salloumet al[33], 2017	Case	ND					ND				
Fruscioneet al[34], 2019	Case	48		22	46		17		20	20	
Marinoet al[35], 2019	Cohort				20					14	
Huet al[36], 2019	Case		54					58			
Leeet al[37], 2019	Case		7	3				8	5		
Limet al[38], 2019	Case	ND					ND				
Wanget al[39], 2019	Case			29	19				48	44	
Chonget al[40], 2020	Case	47	40	3	1		34	39	12	6	
Mejiaet al[41], 2020	Case	ND					ND				
Rahimliet al[42], 2020	Case	ND					ND				

EH: Extended hemi-hepatectomy; LH: Left hepatectomy; LLS: Left lateral segmentectomy; ND: Not properly described; RH: Right hepatectomy; Seg: Segmentectomy;.

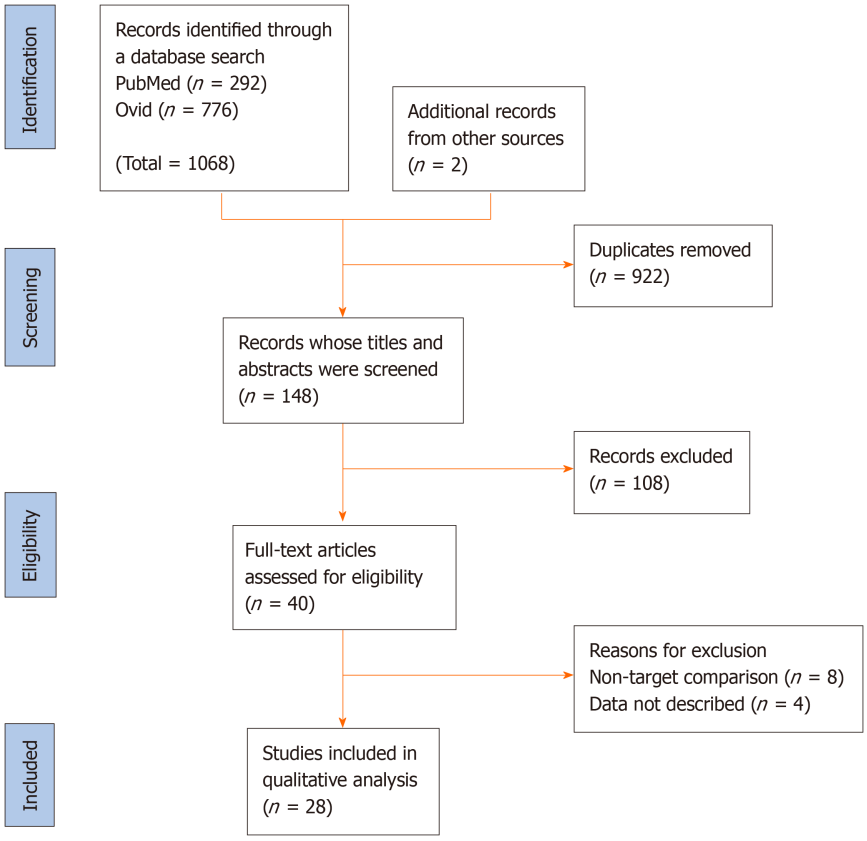


Figure 1 PRISMA flow diagram for this study.

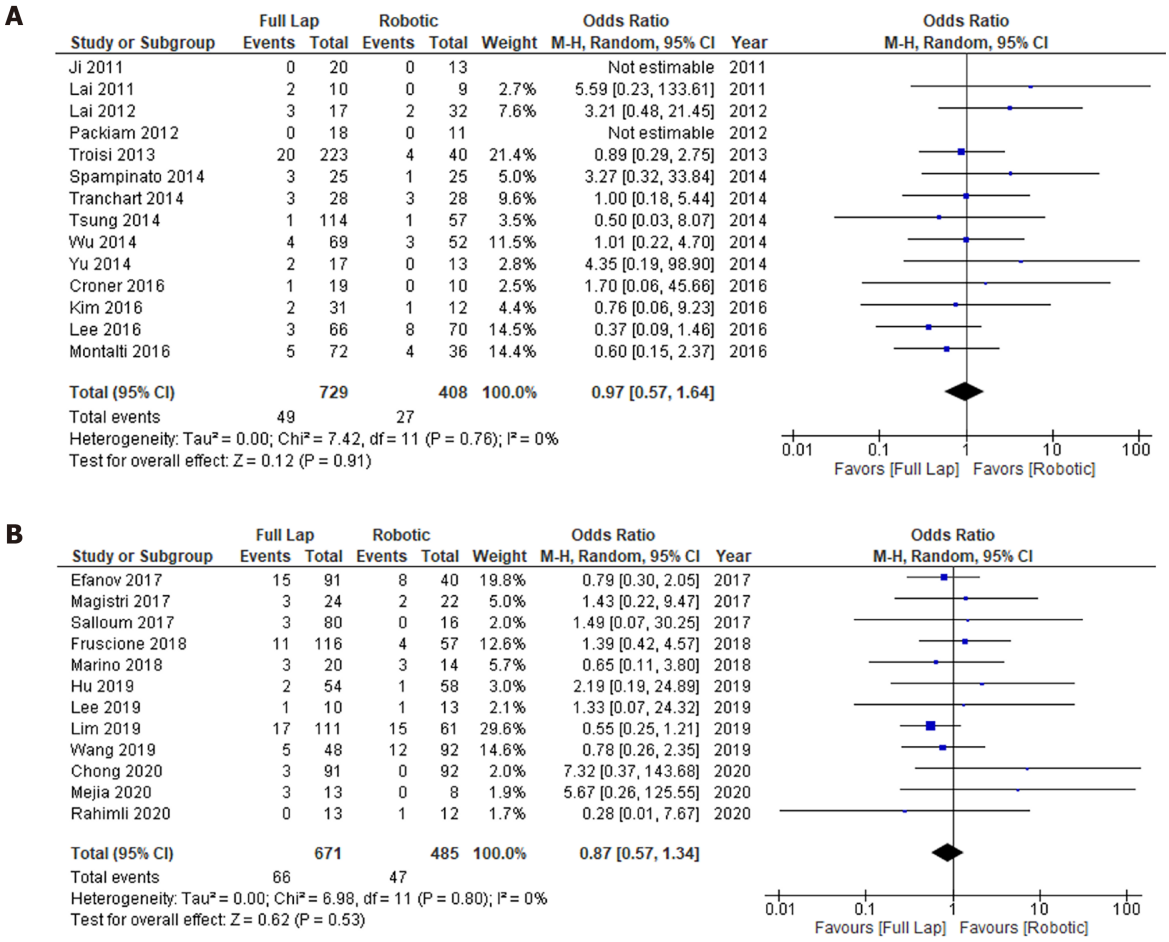


Figure 2 Frequency of Clavien-Dindo grade 3/4 complications. A: 2010-2016; B: 2017-2020.

tional studies. The types of liver resection performed did not differ significantly between the early (Table 1) and recent (Table 2) studies.

Frequency of Clavien-Dindo grade 3/4 complications

The data regarding complications of grade ≥ 3 according to the Clavien-Dindo (CD) classification are shown in Figure 2. There was no significant difference in the frequency of such complications between LLR and RLR in the early or recent studies. Scores of I^2 in both analyses were 0%, which indicated no heterogeneity. The funnel plots were shown in Supplementary Figure 1.

Intraoperative conversion rate

The data regarding the intraoperative conversion rate are shown in Figure 3. There was no significant difference in the intraoperative conversion rate between LLR and RLR in the early or recent studies. Score of I^2 in the early studies was 20% and the one in the recent studies was 44%. The heterogeneities were acceptable in the both analyses. The funnel plots were shown in Supplementary Figure 2.

Intraoperative blood loss

The data regarding intraoperative blood loss are shown in Figure 4. Although LLR tended to cause less intraoperative blood loss than RLR in the early studies, no marked difference in intraoperative blood loss between LLR and RLR was seen in the recent studies. Scores of I^2 in the early and recent studies were 88% and 94%, respectively. Severe heterogeneities were observed in both the early and recent analyses. The funnel plots were shown in Supplementary Figure 3.

Operation time

The data regarding the operation time are shown in Figure 5. Although in the early studies the operation time of LLR was significantly shorter than that of RLR ($P < 0.0001$), there was no significant difference between the operation times of LLR and

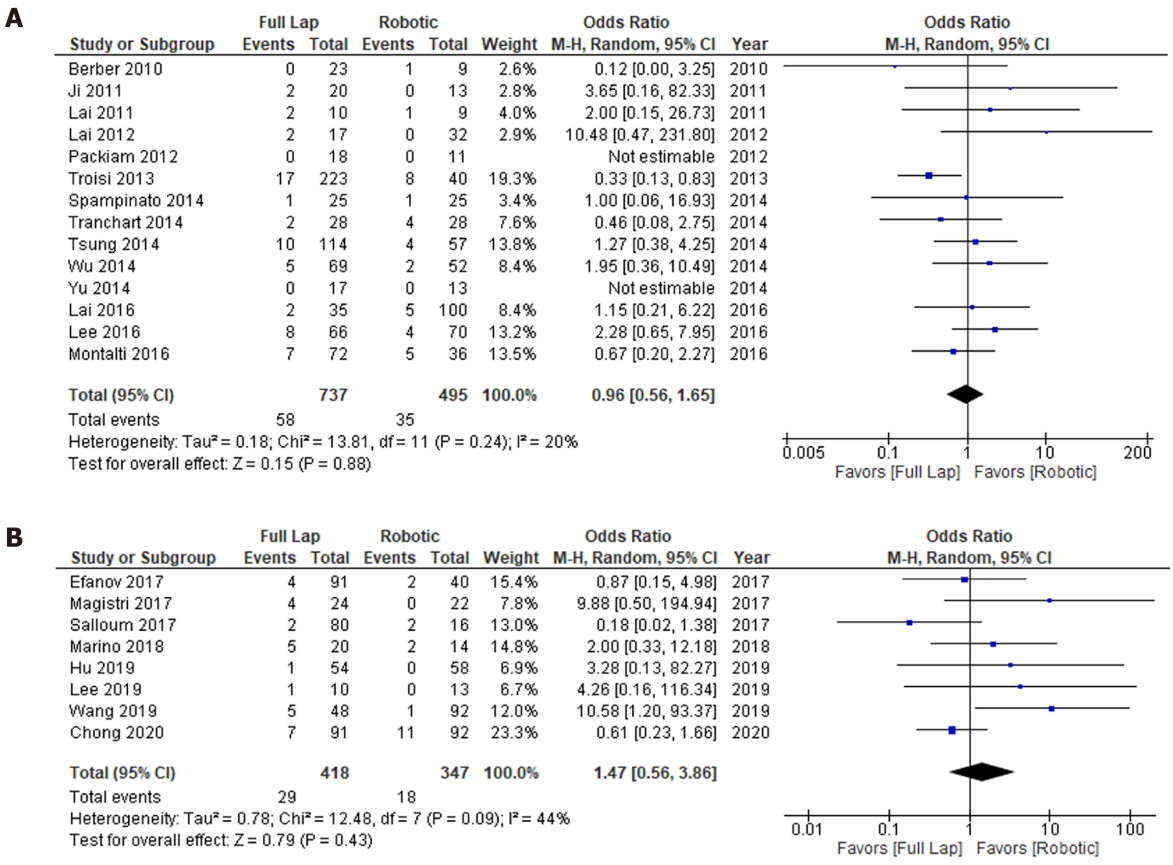


Figure 3 Intraoperative conversion rate. A: 2010-2016; B: 2017-2020.

RLR in the recent studies. Scores of I^2 in the early and recent studies were 81% and 93%, respectively. Severe heterogeneities were observed in both the early and recent analyses. The funnel plots were shown in Supplementary Figure 4.

Quality assessment of the bias

The quality assessment was conducted using the NOS score (Supplementary Table 1 and 2). There was no significant difference in the NOS score between the early and recent studies, although the quality of the studies varied. Summary of the publication bias in each analysis was shown in Supplementary Table 3.

DISCUSSION

MIS has become the standard approach for liver resection[1,4]. The initial disadvantages of RLR were that it involves large amounts of intraoperative blood loss and a long operation time. The recent studies examined in this review indicated that these initial disadvantages have been ameliorated. This finding strongly indicates that a new era of MIS may be upon us.

The CD classification is the standard grading system for surgical complications[43]. The definitions of the grades in the CD classification are based on how the complications are managed, *e.g.*, with pharmacological interventions, surgical interventions, or intensive care. These are indirect signs of complications. Furthermore, the grading system is divided into 5 grades plus 2 sub-grades. We did not find any difference in the types of complications encountered according to the CD classification between LLR/RLR or the early/recent period. This may have been because the CD classification is not suitable for identifying differences between clinical studies due to its use of indirect definitions and a relatively large number of grades. Ideally, surgical complications should be analyzed based on direct symptoms of the actual complications and a simple grading system[44].

LLR and RLR exhibited similar intraoperative conversion rates in both periods. The background data for each study varied, as they were all observational studies. The selection criteria for LLR and RLR were also unclear. Therefore, we could not conclude

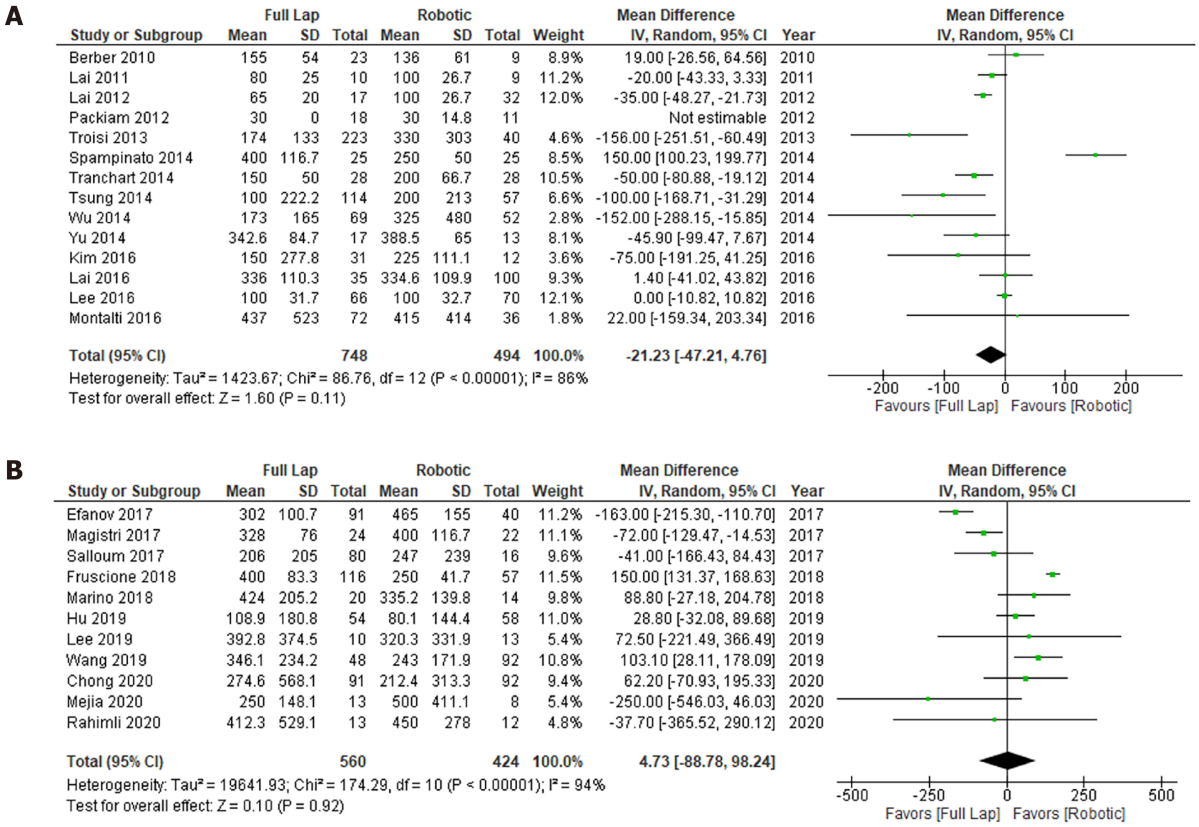


Figure 4 Intraoperative blood loss. A: 2010-2016; B: 2017-2020.

which type of surgery was safer. The maximum intraoperative conversion rate of LLR was about 25% among the recent studies. The maximum intraoperative conversion rate of RLR was about 20% among the early studies, although the mean conversion rate was < 10% in both study periods. In future, these rates could be used as standard clinical goals in order to ensure that surgical quality is maintained.

In the early studies, LLR tended to result in less intraoperative blood loss than RLR, although no marked differences in intraoperative blood loss were seen between LLR and RLR in the recent studies. Several strategies can reduce blood loss during pneumoperitoneum, such as using the head-up position, inducing a high peritoneal pressure, reducing the intratracheal pressure to increase the respiration time, reducing the respiratory volume, using a low central venous pressure, and employing inflow blood control based on the Pringle maneuver[2,45,46]. In addition, it is easier to change the body positions of patients during LLR than during RLR, which could help to control bleeding from veins. Various hemostatic devices are available, such as ultrasonic dissectors, and various hemostatic surgical devices were used for RLR in the recent studies, which may have counteracted the positional disadvantages of RLR. In addition to technical improvements associated with experience, various surgical devices can be used to reduce blood loss during RLR.

In the early studies, the operation time of the RLR was longer than that of the LLR. This is reasonable because it takes time to install robotic towers for robotic procedures. However, the difference in the operation time between the surgical procedures disappeared in the recent studies. It could be that the surgeons became familiar with the robotic procedures, which reduced the time required to set up the robot. Visual support and human-error-canceling functions could also have reduced the operation time[8]. Therefore, the initial disadvantages of RLR have recently been ameliorated.

One advantage of RLR is that it can be used to approach the dorsal segment and caudate lobe of the liver[47,48]. In addition, RLR is superior to LLR for bile duct reconstruction[49]. Therefore, separate tumor location- and surgical procedure-dependent indications need to be developed for RLR and LLR. The differences in the cost and quality-of-life outcomes of RLR and LLR should also be elucidated in the future.

This study had several limitations. First, all of the included studies were observational studies, and no RCT were identified. In addition, the indications for each procedure were not described clearly. The number of subjects recruited for each study

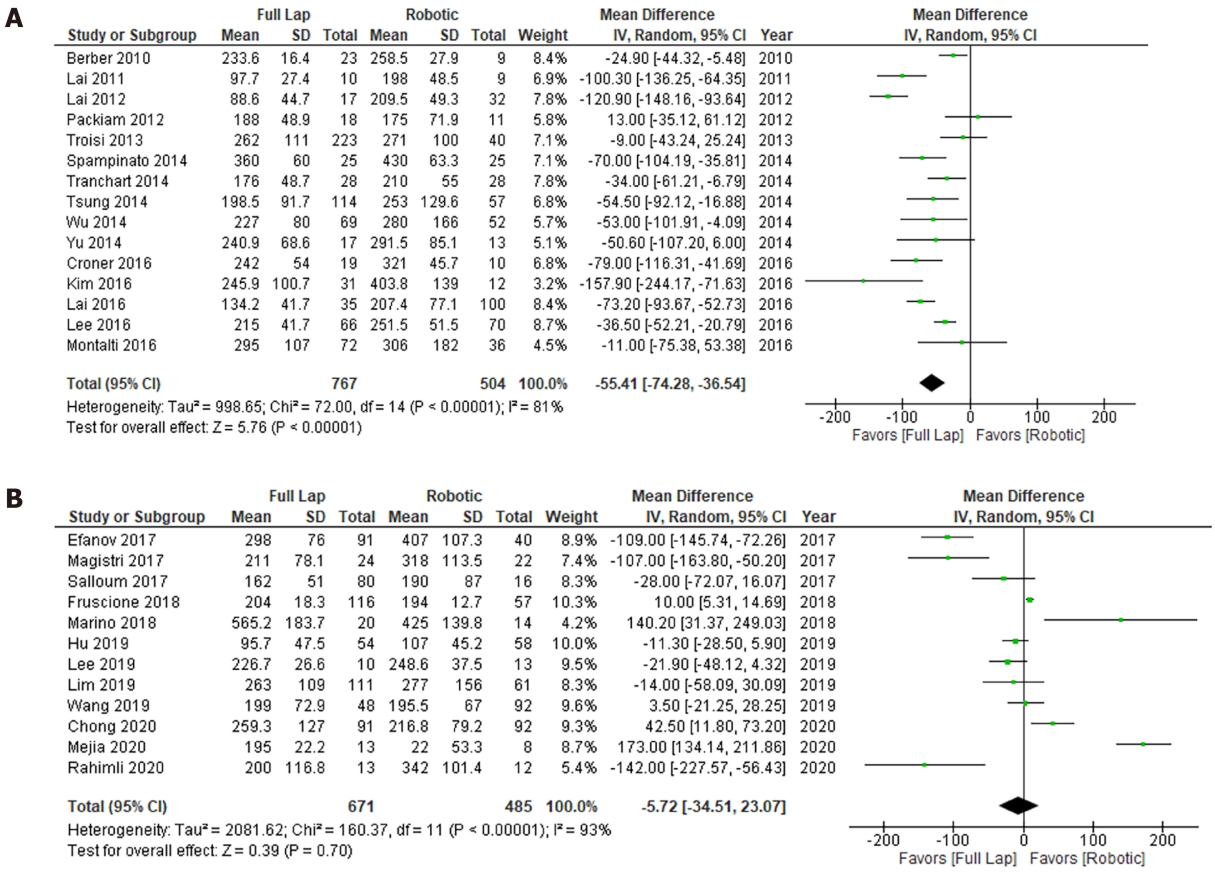


Figure 5 Operation time. A: 2010-2016; B: 2017-2020.

varied, as did the quality of each study. In addition, the clinical backgrounds of the studies differed. Although a few studies involved prospective protocols, at present there is no international registration system for such studies.

CONCLUSION

In conclusion, the initial disadvantages of RLR have been ameliorated. The clinical outcomes of LLR and RLR are comparable. Separate indications for each approach should be developed based on their cost and quality-of-life outcomes. A reliable international registration system for such cases needs to be established.

ARTICLE HIGHLIGHTS

Research background

Robotic liver resection (RLR) has emerged during the last decade. But the clinical outcome of the RLR has been debated.

Research motivation

Clinical outcomes among the laparoscopic liver resection (LLR) and RLR should be compared regarding merit and demerit.

Research objectives

The objective of this study was to conduct a systematic review and meta-analysis comparing the clinical outcomes of LLR and RLR over two 5-year periods.

Research methods

A systematic literature search was performed using PubMed and Medline, including the Cochrane Library.

Research results

A total of 25 articles were included in this meta-analysis after 40 articles had been subjected to full-text evaluations.

Research conclusions

The initial disadvantages of RLR, such as its long operation time, have been overcome during the last 5 years. The other clinical outcomes of RLR are comparable to those of LLR.

Research perspectives

The cost and quality-of-life outcomes of RLR should be evaluated in future studies to promote its routine clinical use.

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 2308-3840 (online)

World Journal of *Meta-Analysis*

World J Meta-Anal 2021 December 28; 9(6): 474-597



Published by Baishideng Publishing Group Inc

WJMA

World Journal of Meta-Analysis

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World Journal of Meta-Analysis

Bimonthly Volume 9 Number 6 December 28, 2021

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INDEXING/ABSTRACTING

The *WJMA* is now abstracted and indexed in China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Hua-Ge Yu*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jin-Lei Wang*.

NAME OF JOURNAL
World Journal of Meta-Analysis

ISSN
ISSN 2308-3840 (online)

LAUNCH DATE
May 26, 2013

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Saurabh Chandan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2308-3840/editorialboard.htm

PUBLICATION DATE
December 28, 2021

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World J Meta-Anal 2021 December 28; 9(6): 474-487

DOI: 10.13105/wjma.v9.i6.474

ISSN 2308-3840 (online)

FIELD OF VISION

Simulating the mind and applications – a theory-based chance for understanding psychic transformations in somatic symptom disorders

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Conflict-of-interest statement: All authors declare no conflict of interest regarding our manuscript.

Country/Territory of origin: Austria

Specialty type: Psychiatry

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

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Abstract

With the new category of somatic symptom disorder/bodily distress disorder in ICD-11, research into pathogenetic and therapeutic pathways is stimulated. By turning away from the definition of somatoform disorders as "the lack of something physical explaining everything", this new classification might offer a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. Modelling and simulation have a long history in science to gain insight also into complex phenomena. Considering the evolution of precision medicine many different parameters are meanwhile operationalised and ready for consequent process research. Calculation models have to fit to the complexity of this disorder category. In an interdisciplinary discourse between computer and medical/psychoanalytic scientists a multilayer, fine grained calculation model is elaborated. Starting from a clinical case history, within iterative discussion, by acknowledging the demand for interdisciplinary synergy and cooperation in science, psychoanalytic theory served as the basis for computer-scientific information technique. A parallelisation with the Mealy model helped to establish a meaningful calculation possibility for further process research. How psychic transformations can be understood properly in order to provide meaningful treatments, the respective training, and to conduct appropriate process- and outcome-research is established

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): E

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Received: August 31, 2021
Peer-review started: August 31, 2021
First decision: November 8, 2021
Revised: November 15, 2021
Accepted: December 24, 2021
Article in press: December 24, 2021
Published online: December 28, 2021

P-Reviewer: Odhar HA, Somasundaram E
S-Editor: Liu M
L-Editor: Kerr C
P-Editor: Liu M

in simulating the mind and applications.

Key Words: Psychic transformation; Computer-technology; Simulation; Somatic symptom disorder; Bodily distress disorder; Mealy model

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Core tip: For the sciences of the mind, especially to understand psychic transformations, a profound interdisciplinary discourse is necessary to bridge the gap between the brain–mind interface, the physical and the information technology fields. The Mealy model guarantees an exact merging of the neurological and the mental domains according to strict scientific principles. The domain of somatic symptom disorders offers a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. To understand psychic transformation, to simulate pathogenetic and therapeutic pathways, the simulating the mind and applications model is helpful to provide further process- and even translational research.

Citation: Löffler-Stastka H, Dietrich D, Sauter T, Fittner M, Steinmair D. Simulating the mind and applications – a theory-based chance for understanding psychic transformations in somatic symptom disorders. *World J Meta-Anal* 2021; 9(6): 474-487
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/474.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.474

INTRODUCTION

Diagnostic concepts: A reconceptualisation of somatic symptom and related disorder

The new category of somatic symptom disorder/bodily distress disorder replaces somatoform disorder (ICD-10, F45.0) and neurasthenia (ICD-10, F48.0)[1]. Hypochondria remains a separate category. With the new ICD-11 category for bodily distress disorder (ICD-11 MMS)[2], the threshold for the diagnosis of disorders with burdensome somatic concerns has been lowered. This might be justified by the fact that single symptoms can be as distressing as multiple symptoms[3]. In the ICD-10 classification, the central feature of the definition was the one that in somatoform disorder the symptoms are not explained by physical causes; this has now been changed. Excessive attention on the nature and progression of a medical condition contributing to a symptom, not alleviated by appropriate diagnostics, also can be classified as bodily distress disorder (ICD-11-Guidelines)[3]. Thus, the reaction of the mind to bodily symptoms is acknowledged to be connected to the body itself. Relevant for the diagnosis are also duration (at least several months) and impairment in important areas of functioning.

By turning away from the definition of somatoform disorders as the lack of something (*i.e.*, the lack of a medical condition explaining everything), this new classification might offer a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. This change in classification manuals also can serve as an opportunity for treatment and process and outcome research.

From a case history and psychoanalysis to artificial intelligence models: Acknowledging the demand for interdisciplinary synergy and cooperation in science

Defining factors to be considered to understand and develop therapies for somatic symptom disorder from an interdisciplinary point of view is one of the steps necessary for developing adequate artificial intelligence models, again rendering research. However, getting started in clinical research often begins with a question arising from a clinical case leading to a hypothesis. The following case report illustrates the complexity of the disorder and how a symptom in this patient diagnosed with somatic symptom disorder causes disproportionate levels of stress. Clinicians dealing with

patients affected by a somatoform disorder are used to dealing with a very heterogeneous syndrome rather than a distinct disease. This complexity often makes it difficult to study treatments and predict prognosis. Much discussion focuses on how artificial intelligence models could contribute to predicting therapy outcomes and simulate the therapeutic processes and effects of different interventions including delayed treatment effects. Thus, the second part of this paper will focus on the presentation of such a model and these aspects.

CASE REPORT

FS is a 52-year-old newspaper carrier working night shifts, who lives with his wife; they have three children. FS was referred from the pain clinic to the psychiatric clinic (tertiary centre, Medical University of Vienna) after 36 years of chronic pain and visiting multiple physicians (general practitioners, neurologists, anaesthetists, and orthopaedists) with the same health problem without any somatic explanation despite a repeated and thorough examination of systems – no therapy had worked. His present complaints are pain throughout the body, especially in the joints, the scalp, (papillary) breast. Furthermore, he complained about gingival paraesthesia, difficulties swallowing, digestive problems, and chronic sleep disturbances. FS is talkative, self-confident, friendly, and outgoing. At the age of 16 years, he met his first love, his admired wife-to-be, and he had his first pain attack, a terrible headache. As a child of divorced parents, he had been separated from his brother and was raised by his mother, grandparents, aunt, and in foster care. In his childhood, FS's emotional and physical needs were consistently ignored, being beaten, and emotionally neglected in foster care. However, he took advantage of the laissez-faire style of education, truanted from school, and stayed out late in the evening. He did not finish clarinet education and began his work as a newspaper carrier, to finance a house and his family. For about 2 years, he suffered from a new kind of fierce pain attack, in his description evocative of this first attack, only deeper and affecting the whole body like "a flash resulting in a pillar of pain from head to toe". The attacks appear about three times a month, in his free time. However, the most annoying complaint is recurrent left-sided hemiplegia in the morning, preventing him from starting the day without the help of his wife. Thus, only after his wife helps him out of the bed, he walks with a limp to the bathroom and takes an extensive shower, with the hemiplegia remitting spontaneously. FS has no explanation for his symptoms and behaviour. However, he had sought comfort in explaining them with the lunisolar tide, with changes in the weather pattern, and with tension and disconnection in family relationships. The relationship with his wife had worsened lately, together with his sexual disturbances. Due to the marital conflicts, he now is motivated for a new check-up and appears excited about any possible help[4].

OVERCOMING THE FOURTH NARCISSISTIC OFFENCE – THE CHANCE FOR AN EFFICIENT COOPERATION

Narcissistic personalities show an extreme over-reaction if confronted with a minor offence, because their grandiose views of themselves, built as a defence against any feeling of vulnerability, are threatened. Unlike a person with narcissistic personality disorder, FS acknowledges him needing help but seems prone to idealising his new saviour, which can quickly turn into devaluation when feeling vulnerable or threatened. For the physicians and therapists now confronted with FS's chronic condition, this means to handle insecurities and to build a therapeutic relationship stable enough to endure expectable alliance ruptures, should the solution to FS's problem not be straightforward. Due to the complexity of the somatoform/somatic symptom disorder, understanding is challenging and often requires the acceptance of not being able to know every detail but to accept the subjective burden of the patient, without giving up on trying. This implies the acceptance of limitations and a confrontation with the gap between ideal and reality. With this reasonably foreseeable loss of the feeling of omnipotence, a narcissistic wound for patients and doctors might result in the feeling of being hurt with a tendency of withdrawal resulting in a repairable interpersonal rupture. Also, interdisciplinary collaboration, needed in clinical work and research when dealing with such complex diseases, can be challenging as it requires a realistic view of one's limitations to participate in a

dialogue with experts and acknowledge contributions to the research topics from other scientific and clinical fields. Nevertheless, diversified collaboration might shed light on this complexity to reach possible treatment strategies, as well as strategies towards precise understanding and process-outcome research. But a closer differentiated look is necessary.

THEORETIC BACKGROUND: THE SOMATIC DISTRESS DISORDER

"[…] The two foes of human happiness are pain and boredom." (Schopenhauer A)[5].

The somatoform pain disorder/somatic symptom disorder/bodily distress disorder is characterized by severe, persisting chronic pain, with a marked psychological strain and pronounced reduction of the patient's quality of life. Furthermore, its origin as well as its overall longitudinal symptom dynamic and severity are linked to emotional conflicts and psychosocial risks and distress. With a prevalence of 9%–20% in the general population, somatoform/somatic symptom disorders' relevance for the health care services is quite high as patients usually are heavy users of services[6-8].

Pathogenesis

Psychiatry and neurology are both sciences dealing with disorders concerning the brain–mind interface. Exploring the interdependence of psychological and biological phenomena, either in a conscious or in an unconscious way, again, might influence both biological and psychological processes, and their interrelations (*e.g.*, psycho-neuroimmunology: depression and different profiles of the immune system[9]).

Neuronal circuits active in psychosocial distress are also associated with physical pain; emotional and physical pain exist on the same continuum[10,11]. In early childhood, somatisation is regarded as a normal and necessary developmental phase, as a reaction to stress and distress, and diminishes with maturation[12]. The same reaction already in older children is regarded as a pathological one, and the need for a classification that does justice to these developmental steps, thus avoiding over-pathologising, has been highlighted[13-15]. Interpersonal and intrapersonal emotional distress is felt as agonising pain and suffering instead. In such patients, adequate ways of communicating distress are not well developed; dysfunctional interpersonal affect regulation between caregiver and child is at the origin of the disorder[12]. On a neuronal level, networks associated with interpersonal distress are also associated with the neuronal circuits responsible for pain[16-18].

Development of affect regulation

However, at the core of the problem are dysfunctional affect regulation abilities and attachment patterns, developed in early interpersonal experiences, thus being transmitted from one generation to the other[12]. Especially in vulnerable persons with genetic predisposition and in unfavourable environmental conditions and contexts such deficits might lead to somatoform disorders[12]. In the above-mentioned case report, the educational style was characterised by emotional neglect and rejection. FS developed rejection sensitivity and a high level of worry[19]. His discomfort with closeness shows up in the relationship with his wife. He is caught in repetition: his pain expression, an expression of his distress and inner tension, leads to his wife having to care for him. Thus, in a way, he is asking for closeness, but at the same time, it seems that he feels uneasy about his wife caring for him.

While in early childhood, especially in the perinatal period, arousal and excitation are experienced and regulated somatically[12], later more sophisticated ways of regulating external and internal stimuli should rise, together with the development of cognition and higher mental functioning.

As separation from the caregiver might provoke fear in early childhood, this is one of the challenges to master in this developmental period. Diverse strategies and ways to deal with this and similarly distressing events (*e.g.*, hunger, tiredness, pain or fever) need to be developed, partly depending on the availability of care). In psychodynamic theory this process especially has been investigated in Winnicott's theory of holding (importance of the external object), Bion's theory of container/contained, and Klein's theory with a particular focus on the internal and phantasmatic experience of the child [20]. At first, the child needs soothing and satisfaction of needs by the caregiver in case of distress (compare Figure 1).

Additionally, postevent processing of distress varies and is relevant (see[21] for social distress). However, with time, it learns to soothe itself together with an understanding of the situation together with its own and the others' affective and

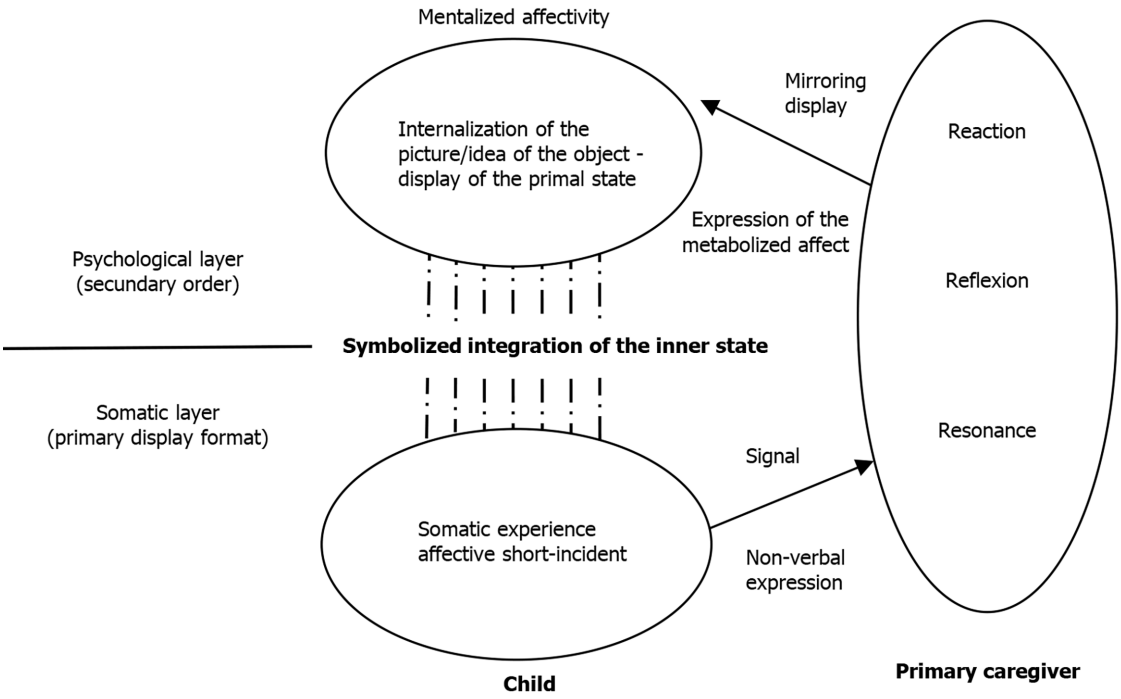


Figure 1 The emergence of mentalized affectivity. Mutual interactions between caregiver and child are shown as a process where the caregiver infers the inner thoughts and affective states through signals expressed by the child (and the contextual information and knowledge available). At first, the child notices a somatic experience and expresses it nonverbally to the caregiver as a signal. Then, the caregiver metabolises this signal (resonance, reflection, reaction) and communicates an expression of this metabolized affect to the child (mirroring display). In the next step, the child internalises this reaction of the caregiver in response to its own experience as a picture/idea of the primal state. In such a way a transformation from a somatic experience to an integration of the child's inner state is acquired *via* symbolisation in repeated social interaction.

mental states. Intention mirroring was found to be more frequent in securely attached mothers, based on well-attuned, affect-mirroring communication[22]. Self-compassion has been shown to be lower in patients with somatoform symptoms when confronted with healthy subjects[23]. In perpetuating the somatoform disorder, a learned bias to shift one's attention on somatic/bodily processes leads to an exaggerated experiencing and perception of somatic signals, increasing the risk of misperception and misinterpretation of such sensations. Furthermore, also a link between attachment patterns and somatisation has been shown for adults (insecure attachment[22,24], fearful and preoccupied attachment[25]). The case history discussed earlier in this paper also shows how "doctor shopping" can be perpetuated by the patient's insecure attachment pattern and the need for the symptom as a sign and symbol, with high costs for the health care system and a multitude of examinations for the patient.

To conclude, affect regulation is linked to the attachment pattern and mediated by social interaction (see also[12], compare Figure 2). An insecure-avoidant attachment pattern is associated with problems to trust someone and the intolerance of closeness [26]. Thus, interventions addressing attachment in therapy have the potential to improve symptoms.

Predisposing factors for somatoform disorders/somatic symptoms/bodily distress disorders

A higher lifetime prevalence of somatoform disorders has been observed for individuals with early trauma[27], lacking parental care[28], negative experiences/stressful live events[29,30], and child abuse[31]. However, the manifestation of a somatoform disorder also depends on the parenting style[28] (*e.g.*, avoidance/rejection, conflicts or emotionally unstable parents, lack of discipline and inconsistent discipline, or controlling and overprotective parents)[28]. Continuity of somatic symptoms between adolescence and early adulthood has been shown and points to the high tendency of chronicity in this group of patients. Earlier research has shown that the level of somatic symptoms in a patient's parents might be associated with the ones in their children [32,33]. In twin studies, a genetic predisposition was suggested[34].

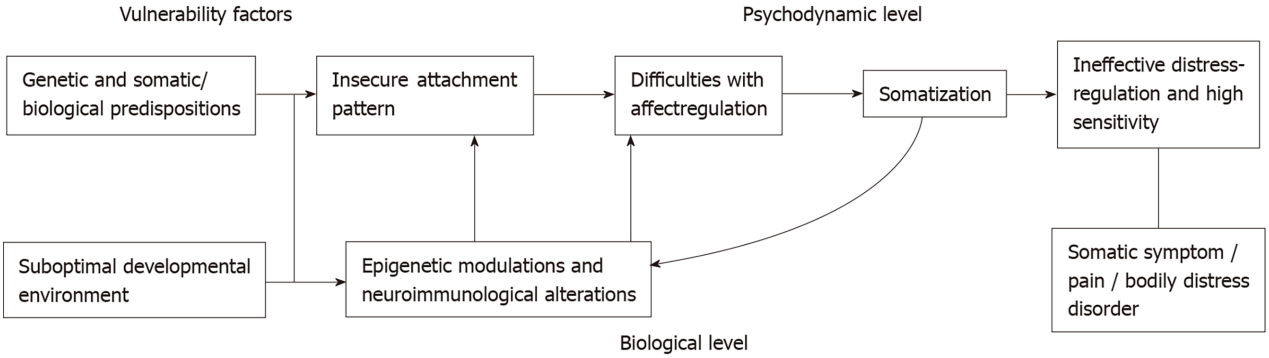


Figure 2 Vulnerability and psychodynamics.

Biological perspectives on somatoform disorders

High subjective distress, in severity not fitting the present objective findings, is one of the main symptoms of somatoform disorders (ICD10)[35]/somatic symptom/bodily distress disorders (ICD11)[2]. Unfortunately, although somatoform disorders encompass a heterogeneous group of diseases, some unrecognised diseases, misclassified as somatoform disorders might be relabelled after a repeated, sufficient examination. Furthermore, some potentially curable diseases still might lack the techniques to study them[36].

As somatoform symptoms are not considered strictly mental/psychological events, biological processes play an important role[36,37]. The underlying physiology of distress has been studied, explaining to some extent the genesis, and the experiencing of symptoms[36]. Especially research on inflammation-associated symptoms has gained promising results lately. The association between subjective health complaints and inflammation has been shown[38,39]. Raison and colleagues[39] have argued that depression is an accessory phenomenon that comes together with adaptive advantages due to genes promoting inflammation. The field of psychoneuroimmunology investigates environmental influences on the development of the immune system. Repeated exposure to danger leads to immune reactions (proinflammatory state) with pro-inflammatory cytokines and the possibility of immune sensitisation. For somatoform disorders and for sickness behaviour, specific cytokine patterns have been identified [40].

Social exclusion has been shown to be associated with somatic complaints, especially with pain. Social rejection and physical pain both are distressing, and they share a common somatosensory representation[41]. Experiments investigating the pain threshold in children/adolescents with somatic symptoms showed a divergent reaction regarding their sensory threshold after social exclusion when contrasted with controls[42]. While the group with somatisation showed a stable sensory threshold, the controls showed a decreased threshold[42]. Adolescents with functional abdominal pain showed increased parasympatic activation when exposed to induced social exclusion, whereas healthy controls showed no such activation pattern[43].

As mentioned above, the pain network is linked to the neuronal network concerned with the regulation of distress provoked by interpersonal stimuli. Important structures involved in distress regulation (emotional and the affective component of somatic pain) are the medial prefrontal cortex (antinociceptive effects, including biopsychological pain management but also chronification[44]), the dorsal anterior cingulate, and the anterior insula[41]; these regions are also involved in processing social rejection. While the brain regions concerned with the somatic representation of physical pain include the operculo-insular region; these regions are not activated by social rejection[41].

As pain is associated with depression and antidepressants influence pain (*e.g.*, tricyclic antidepressants such as amitriptyline, and selective serotonin reuptake inhibitors such as citalopram), common biochemical mechanisms are likely[45].

The role of oxytocin in social cognition, including the development of attachment and trust as well as in pain has been investigated, but there are still many open questions[46].

Exogenous factors influence human life and health. Evidence suggests that social interactions might have an influence on the expression of genes[47,48], while epigenetic and genetic predispositions modify the response to environmental factors [48,49]. Epigenetic changes have been shown to be influenceable by separation and

traumatisation with potentially permanent and profound changes. Mechanisms underlying epigenetic changes include DNA methylation, histone modification, RNA silencing, regulation of genes, *etc.*[49]. For example, epigenetic changes are one key mechanism of how stressors interact with the genome[50]. Influences on the reaction to stress in patients with depression have been shown to be associated with a changed expression of cortisol receptors in the hypothalamus (methylation and modification of histones), leading to a prolonged and pronounced stress reaction due to higher cortisol levels (changed glucocorticoid signalling). The hypothalamic–pituitary–adrenal axis activation is common in major depressive disorder[51].

Reconsidering the main aspects from the biological, psychological, developmental, and social domains it often remains open, how psychic transformations can be understood properly to provide meaningful treatments, the respective training, and to conduct appropriate process and outcome research.

UNDERSTANDING PSYCHIC TRANSFORMATIONS

One problem in psychotherapy research is finding answers to the questions of how psychotherapy works, and what works for whom, how do psychic transformations happen at all, and how to make them last? Patients requiring psychotherapy are a heterogeneous group (differences in medication, social context, comorbidities, previous therapy, age group, *etc.*), this makes interpretation of data difficult in investigations with small sample sizes. It is not possible to run the same experiment in the same patient twice under different environmental or social conditions, because each intervention changes the investigated individuum and context. Investigating long-term data is expensive, but long-term results matter as much as process research does. Comparison of new therapeutic approaches with the best known and best available care is particularly problematic in long-term therapies with real patients, because of good enough evidence for existing therapies. Randomisation to new therapeutic approaches and innovative interventions is problematic due to ethical concerns (potentially unknown side effects and unknown long-term outcome, freedom of choice for the paying and informed patient).

SIMULATING THE MIND AND APPLICATION

Modelling and simulation have a long history in science to gain insight into complex phenomena and conduct virtual experiments when real ones are not possible. This approach could be useful here. Artificial intelligence models of the mind–brain interface, rending exploration of machine learning capabilities possible, also could allow for investigations that are ethically or technically not possible in humans. To that end, the simulating the mind and applications (SiMA) model was developed. Far more than a mathematical toy, it supports the exploration of theoretic and abstract concepts so challenging as the connections between psyche and body.

The model of Mealy: bridging the gap

Coupling of the neurological system and the psyche: When searching for a scientific description of a model that considers a coupling of the neurological system and the psyche, one is inevitably confronted with the contradictions in the nomenclature of the different scientific communities. When we use the term "brain", we often hear the accusation that parts of the nervous system, as well as the associated sensory and actuators systems, are being excluded. The term "mental apparatus" is quickly pushed into the corner of neurological reductionism, and the term "nervous system" is hardly associated with the psyche. Therefore, for the purpose of this article, the term Ψ-organ will be used. The term "organ" indicates that the information system of the human being can be regarded as a unified system like all other organs. The term "Ψ" indicates that mainly psychoanalytical knowledge is used to describe the psyche of the Ψ-organ.

With this foundational understanding, the challenging scientific question of how the physical and the psychological can be described without contradiction in a common and unified model can be posed.

There have been a few approaches to this question in the past; all of which have been unsatisfactory. Two of the most frequently cited are that of Peterfreund and that of Turkle[52,53]. Peterfreund was too focused on the mathematical considerations and barely made the connection to neurology. He did not achieve a complete model based

on neurological as well as psychological concepts[52]. Turkle did not find a unified model between neurology and psyche, either, but focused mainly on the collaboration between artificial intelligence and psychoanalysis[53]. A relatively new idea from Solms[54] must be viewed sceptically from an information technology (computer technology) perspective. His attempt to build a bridge between the mental and neurological description is based on the assumption that one can merge the methods and laws of physics with those of information technology in one mathematical equation system, without substantiating this experimentally.

The model concept of SiMA is different. Dietrich uses Mealy's theory, which is generally applied in computer science, to bridge the gap between the physical and the information technology fields[55,56]. It guarantees an exact merging of the neurological and the mental domains according to strict scientific principles. The experimental simulation results confirm his approach.

Mealy published his idea in 1955[57]. He succeeded in developing a modelling method named after him for the calculation of electronic digital circuits. The digital circuit is transferred into a two-layer model. The lower layer contains the functions that are described physically, while the upper layer contains the functions that are described in terms of information technology (Figure 3). Both layers are connected *via* a clearly described interface. The description is a mathematical process. It can only be imagined abstractly. One must be aware that the real circuit is conceptually split into two layers: the physical layer and the information layer (not comprehensible from a physical point of view). The interface between both layers is defined by the information flowing through them. In the lower layer, the information is described physically, and in the upper layer, the same information is described information technologically.

The physical layer in Figure 3 can be diverse. In digital electronic circuits, functions can be transistors, resistors, or diodes, and information can be expressed by physical quantities such as voltages or currents. Accordingly, neurons can be described based on such functions. Referring to Figure 3, the information quantities entering the lower layer (information a) are quantities that act on the human sensors and thus must be described physically. The same applies to the output quantities h. The information quantities acting within the lower layer (the information quantities b, c, f and g) are therefore to be described by electrical properties: voltages, currents and temporal behaviour of the neurons. If one changes to the upper layer, *i.e.*, all quantities (the information quantities d and e) become independent of their underlying physics and are described purely in abstract terms. Figuratively speaking, electrical signals turn into bits and bytes. The passage of all information through the functions of the upper layer is also independent of time, it happens instantaneously. If information d in Figure 3 is generated by information c, it is simultaneously present as a quantity at the output of the upper layer and thus as a quantity f at the lower layer.

Mealy and his team were able to prove through experiments that this model – also known as the Mealy machine – was not just a mathematical gimmick. Scientists also developed modified mathematical description methods that went beyond this. Today, every design language for the development of hardware (like computer components), is based on this very abstract principle. Nowadays, no computer is conceivable without Mealy's theory.

A generalization of the Mealy principle is just as important. Imagine that several computers are to communicate with each other. The first international standardised model of this kind was the International Organization for Standardization (ISO)/open systems interconnection (OSI) model. The layer described in terms of information technology can be subdivided as required and the functions it contains can be allocated within it according to their specific tasks. The layers are arranged hierarchically. Each column in Figure 4 represents such an ISO/OSI model. The lowest layer (layer 1) is always the physically described layer, which defines the (physical) connection to the other computers. The upper layers of Figure 4 are the units described in terms of information technology. This means that the upper layer in the Mealy model is subdivided into several, further layers. The basic principle is identical in both models.

The original Mealy model (Figure 3) was developed for the design of digital circuits, *i.e.*, to obtain a holistic, functional model for the physical and informational description. The generalization of this principle, *i.e.*, the abstraction of physical signals and states into information symbols that can be further aggregated and processed on higher system layers, leads to models such as the ISO/OSI model (Figure 4) or, in SiMA, a holistic, functional model of the Ψ-organ.

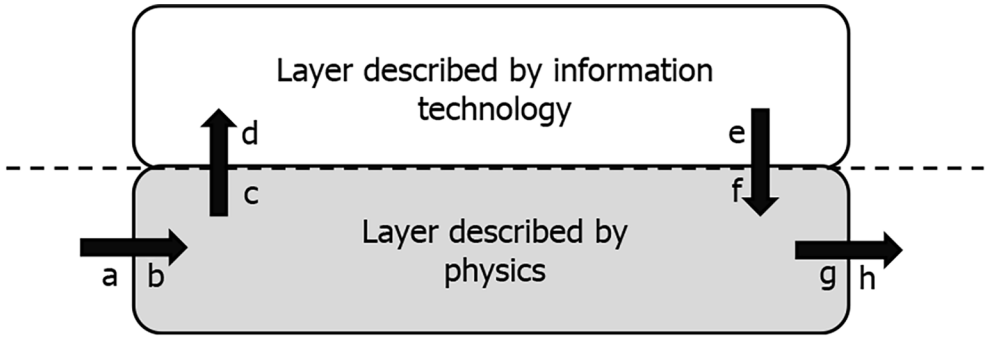


Figure 3 Mealy model. Current state and current inputs determine the Mealy model's output values. A mealy machine consists of a finite set of states, inputs and outputs and a transition as well as an output function. The model produces an output (h) while an input (a) is received (in the same simulation step). In this figure the real circuit is conceptually split into two layers: the physical layer (rectangle shaded in grey) and the information layer (rectangle shaded in white). The interface between both layers (dashed line) is defined by the information flowing through them. An abstract concept of the transition between the two layers (information technology and physics) is shown.

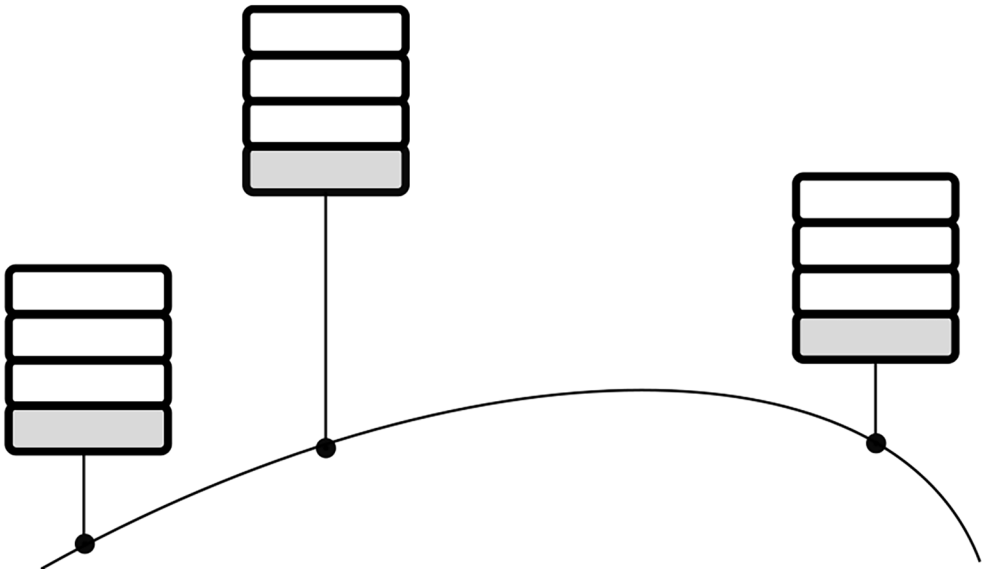


Figure 4 Possible coupling of computers according to ISO/OSI. Each column in this figure represents such an ISO/OSI model with hierarchically arranged layers. The lowest layer (rectangle shaded in grey) is always the physically described layer, which defines the (physical) connection to the other computers. The upper layers of the columns in this figure are the units described in terms of information technology (rectangles shaded in white: the upper layer in the Mealy model is subdivided into several layers as required, with functions allocated within according to the specific tasks). ISO: International Organization for Standardization; OSI model: Open Systems Interconnection model.

To bridge the gap to the Psy-sciences to overcome the fourth narcissistic offense of not being able to understand somatic symptom disorders / bodily distress disorders good enough and provide meaningful treatment options for patients, a precise stepwise collaborative working model must be considered to simulate possible ways of understanding.

SOMATIC PAIN IN SiMA AND ITS POSSIBILITIES FOR BRIDGING THE GAP

In structuring and describing functions of the mind, SiMA builds on psychoanalytic principles and concepts[56]. Functions and their inter-relations are grouped in layers according to their association with physical, unconscious and conscious processing of information. To avoid systematic errors in the development of the technical implementation and to ensure a consistent and holistic model, the concept was developed in an interdisciplinary discourse.

A central point in the model is the feedback from the body of what in SiMA is called an agent. To that end, interfaces for sensory inputs are foreseen[58], and the body of the agent is modelled. The concept of pain, however, was not originally included[59] because in psychoanalysis, pain is only seen as a pseudo drive. "The goal of this pseudo drive is just the cessation of the organic change and the associated unpleasure. Other, direct pleasure cannot be derived from the stopping of the pain. Pain is also imperative…"[60,61]. This served as guideline for including a model of somatic pain. Its implementation was tested *via* simulations, and the results discussed in an interdisciplinary review.

Originally, the SiMA model did not comprise feedback to the psyche when the agent experienced pain. Consequently, the agent would not show a reaction if its health status worsened. The inclusion of the pain concept necessitated an extension of the existing model structure and the inclusion of interfaces across the layers to allow for representing somatic pain in the psyche. The source of pain are sensory inputs of the body signalling a change. In contrast to drives, pain relief cannot generate pleasure. Therefore, pain cannot be treated in the drive track of the model. Rather, it must be included in the perception track and produce corresponding unpleasure there. This unpleasure is used in turn to create rated memories and emotions.

Figure 5 shows the functions added to the model (relevant for the processing of somatic pain[62]). Functions F12 generate the sensory inputs to the body. They register changes, *e.g.*, when the agent is hurt. The sensor value is then transferred to F13 which extracts symbol's that can subsequently be processed by the psyche. These symbols contain actual values as well as connections to the symbol health status. All this information is provided to the mental layer and further processed in F14 which generates a factual perception related to the representation of pain (Figure 6)[62]. The amount and rate of change of the symbols translate into a level of the somatic pain. Moreover, F14 generates unpleasure correlated to the pain level. This unpleasure is factored into the calculation of the emotion and added to the existing unpleasure stemming from the current drive situation. It is thus available to the functional model for subsequent decision-making and memory creation.

Calculation of the unpleasure in F14 accounts for changes in the health status as well as the difference to the optimal health,

$$U_i = (G_{i-1} - G_i) \times G_i + (1 - G_i) \times (1 - G_i)$$

with U_i being the new unpleasure value, G_i the current health status, G_{i-1} the health status from the previous simulation cycle, and $1 - G_i$ being the difference between the current and the maximum health. The health value is from the range [0..1], where 1 represents optimal health and 0 represents the death of the agent. The resulting unpleasure is also scaled to [0..1]. The equation is split up into two parts, where the first part is the change of the current health to the previous state and the second part is the difference between current and the maximum health. Both parts are weighted by the current health status (G_i). If the health status is high, the change of health will be weighted more than the difference to the maximum health. These equations were derived in interdisciplinary discussions and proved reasonable in simulation experiments.

Optimal health and its definition are challenging, as it presents an ideal but subjective state, characterized by the absence of disease but furthermore by being at one's optimum and being balanced in all aspects of existence. Thus, a difference ($1 - G_i$) between this ideal and the current health states (G_i) is the normal state, always including a certain but variable amount of unpleasure (U_i). The tricky part is quantifying the amount of the difference.

THERAPEUTIC OPTIONS

Psychoanalysis and focal psychodynamic-interpersonal therapy

The influence of early mother–child relationships on the predisposition for the manifestation of a somatoform/somatic distress disorder led to a psychodynamic therapy based on this finding. While at the beginning of treatment, understanding, and legitimation of somatic complaints and relaxation training is the focus, later, the differentiation between affects and perception of sensations of the body is trained. Links between affect experience and regulation and symptoms/body perceptions are drawn. The technique uses interpersonal experiences for this approach, a new interpersonal sphere (for transformation from layer 1 to layer 3) is created within the therapeutic relationship. Dysfunctional health attitudes (Layer 3), associated with the symptoms (from body or F12) and the experiencing of body sensations (F14 *via* F13

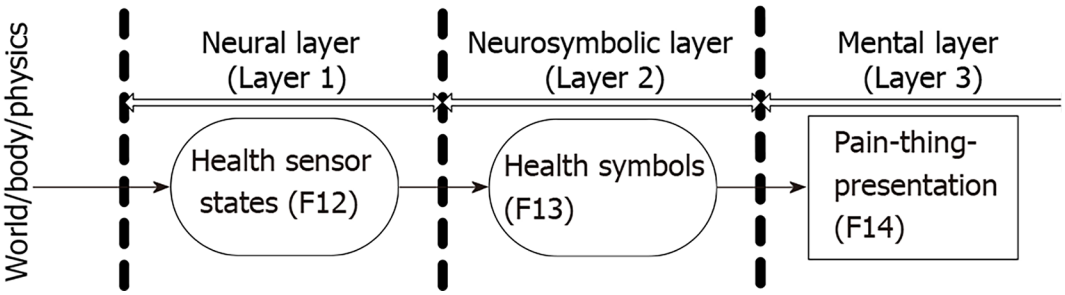


Figure 5 Excerpt from the simulating the mind and applications model. The functions relevant for the processing of somatic pain are shown (marked in dark grey).

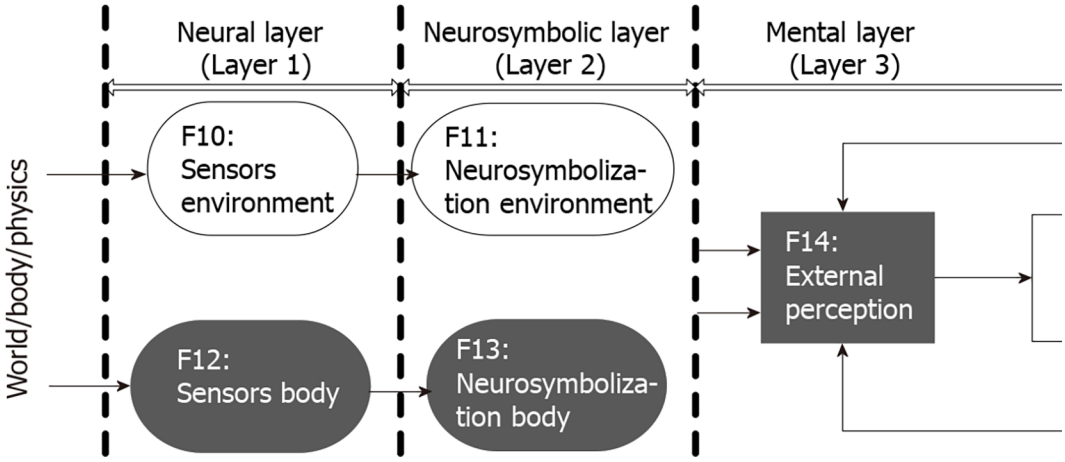


Figure 6 Creation of pain perception from the body sensors of the agent[62] **Neural layer (layer 1): functions F12 generate the sensory inputs to the body (health sensor states/registering changes)**. Neurosymbolic layer (layer 2): this sensor value is then transferred to F13 which extracts symbols, containing actual values as well as connections to the symbol health status. Mental layer (layer 3): all this information is provided to the mental layer and further processed in F14 which generates a factual perception related to the representation of pain (*i.e.*, unpleasure correlated to the pain level).

and F12), are also addressed to consolidate the therapy progress. Complex analysis and interpretations are not the focus of this focal therapy approach. At the end of therapy, the patient gets a letter from the therapist summarizing the content of the therapy. Beginning with the focus the patient then can transfer this re-consolidation and corrective emotional experience to other difficulties in life and can utilize psychoanalysis[63,64].

CONCLUSION

Outlook for further research

To facilitate a second order of meaning and understanding, a symbolized integration of somatic pain has to be established. This is normally provided in interaction circles with the primary caregiver (Figure 1) – and for therapeutic purposes then with the psychoanalyst. The affective signal is affectively marked ("resonance" in Figure 1, in Mealy's model Figure 3 "c"), then mentalized, reflected by the caregiver ("d" in Figure 3 or in psychoanalytic theory "alpha function", "dream work", "reverie"[65-67]) and a reaction is set with a metabolized affect ("f" in Figure 3). Such processes serve as essentials for establishing meanings, words, and understanding symptoms. With the Mealy model, these processes can be operationalised in a meaningful way. As an affectively meaningful second-order layer can arise and develop in a relationship, the second person with their reflective function must be considered. This conceptualisation is saturated with systems-theoretical considerations[68] and is currently empirically investigated (*e.g.*, embodiment literature[69] or epistemic trust investigations[70]).

For further research calculating operationalised psychic parameters is not enough, the weight or underlying structure/function often must be additionally considered. Therefore, the SiMA-Model can give advantages for further meaningful process research. This collaboration and iterative efficient cooperation can serve as a possibility for overcoming the fourth narcissistic offense to facilitate meaningful research for burdened patients and their therapists.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 488-495

DOI: 10.13105/wjma.v9.i6.488

ISSN 2308-3840 (online)

OPINION REVIEW

Is dose modification or discontinuation of nilotinib necessary in nilotinib-induced hyperbilirubinemia?

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Author contributions: Tan YW completed the paper independently.

Conflict-of-interest statement: The author has no conflict of interest to disclose.

Supported by the Social Development Project of Jiangsu Province, No. BE2020775; and Medical Project of Health Department Jiangsu Province, No. H2018021.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Abstract

Nilotinib is a specific breakpoint cluster region-Abelson leukemia virus-tyrosine kinase inhibitor that is used as an effective first- or second-line treatment in imatinib-resistant chronic myelogenous leukemia (CML) patients. Hepatotoxicity due to nilotinib is a commonly reported side effect; however, abnormal liver function test (LFT) results have been reported in asymptomatic cases. When alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are more than five-fold the upper limit of the normal (ULN) or when the serum total bilirubin level is more than three-fold the ULN, dose modification or discontinuation of nilotinib is recommended, resulting in decreased levels of hematological indicators in certain patients with CML. Nilotinib-induced hyperbilirubinemia typically manifests as indirect bilirubinemia without elevated ALT or AST levels. Such abnormal liver functioning is thus not attributed to the presence of a true histologic lesion of the liver. The underlying mechanism may be related to the inhibition of uridine diphosphate glucuronosyltransferase activity. Therefore, nilotinib dose adjustment is not recommended for this type of hyperbilirubinemia, and in the absence of elevated liver enzyme levels or presence of abnormal LFT findings, physicians should consider maintaining nilotinib dose intensity without modifications.

Key Words: Tyrosine kinase inhibitors; Nilotinib; Chronic myelogenous leukemia; Hyperbilirubinemia; Drug induced liver injure; Liver injury

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Core Tip: Hepatotoxicity due to nilotinib is a commonly reported side effect; however, abnormal liver function test (LFT) results have been reported in asymptomatic cases. Nilotinib-induced hyperbilirubinemia manifests usually as indirect bilirubinemia

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Received: December 29, 2020
Peer-review started: December 29, 2020
First decision: October 11, 2021
Revised: October 14, 2021
Accepted: December 24, 2021
Article in press: December 24, 2021
Published online: December 28, 2021

P-Reviewer: Cengiz M, Nabil A
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Liu M

without observation of elevated alanine aminotransferase or aspartate aminotransferase levels. The underlying mechanism may be related to the inhibition of uridine diphosphate glucuronosyltransferase activity. Therefore, in the absence of elevated levels of liver enzymes or presence of abnormal LFT findings, physicians should consider maintaining nilotinib dose intensity without modifications.

Citation: Tan YW. Is dose modification or discontinuation of nilotinib necessary in nilotinib-induced hyperbilirubinemia? *World J Meta-Anal* 2021; 9(6): 488-495
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/488.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.488

INTRODUCTION

Chronic myelogenous leukemia (CML) originates in hematopoietic stem cells of malignant hyperplastic diseases and accounts for 15%–20% of all leukemia cases, with an incidence of 1–2/100000[1]. CML is one of the most common types of leukemia and is marked by the presence of a breakpoint cluster region (BCR)-Abelson leukemia virus (ABL) on the (9, 22)(C34, C11) chromosome translocation fusion gene, *i.e.*, the gene encoding P210-type fusion protein which exerts a type of tyrosine kinase activity. The subsequent abnormal clonal proliferation of myeloid hematopoietic cells is the primary cause of CML.

BCR-ABL tyrosine kinase plays an important role in cell differentiation, division, adhesion, and stress response, and its constitutive activation can lead to the development of leukemia. BCR-ABL is expressed in tumor cells of 95% of patients with CML, whereas it is not expressed in normal cells. Therefore, specific BCR-ABL tyrosine kinase inhibitors (TKIs) can be used to effectively treat CML[2].

TKIs are known to induce hepatotoxicity[3]. A systematic study conducted on the basis of 12 previous studies demonstrated that TKIs are associated with a higher risk of hepatotoxicity, compared to placebos[4]. Regarding hepatotoxicity of grade 3 or higher, the instructions recommend dosage reduction and discontinuation to allow recovery of liver functioning. The objective of this study was to confirm that the distinction between true hepatotoxicity and hyperbilirubinemia alone does not require dosage changes or discontinuation of treatment for hyperbilirubinemia caused by nilotinib alone.

PRESENT STATUS OF NILOTINIB TREATMENT

Nilotinib is a molecularly targeted kinase inhibitor that competitively binds to the platelet-derived growth factor C-kit and ABL kinases, thus it is effective for treating CML. A five-year follow-up survey showed that the 5-year cumulative rate of complete cytogenetic remission was 87%, the estimated 5-year survival rate was 89%, and the rate of progression-free survival was 93%[5]. Up to 98% of the patients achieved hematological remission, and cytogenetic response was achieved in 86% of the patients at the chronic stage after treatment. After 1, 2, 3, and 4 years of continuous treatment, only 3.4%, 7.5%, 4.8%, and 1.5% of the patients, respectively, were expected to experience deterioration. Nilotinib is also considered an effective second-line treatment for imatinib-resistant cases[6,7].

HEPATOTOXICITY OF NILOTINIB

Common adverse reactions to nilotinib include dermatological, digestive, and cardiovascular side effects. Abnormal liver function is a common side effect in nilotinib-treated patients, and a considerable proportion of the patients show elevated levels of liver enzymes and bilirubin, based on analyses of phase II and III trials (Table 1). Up to 70% of the patients exhibit elevated levels of liver enzymes[8], and most patients are asymptomatic, presenting with grade 1–2 abnormal liver function. This typically occurs within 10–14 d of initial treatment, and only 4%–9% of the

Table 1 Incidence of biochemical abnormalities across the second-line and frontline nilotinib trials

Patients (%)	Second-line trials				Frontline trials							
	Phase 2		Phase 3		Phase 2				Phase 3			
	2101[35] (*n* = 321)		ENACT[36,37] (*n* = 1422)		MDACC[38] (*n* = 61)		GIMEMA[17] (*n* = 76)		ENESTnd[39] 300 mg twice daily (*n* = 279)		ENESTnd[39] 400 mg twice daily (*n* = 277)	
	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4
Bilirubin elevation (total)	72	7	25	4	39	7	53	16	53	4	62	8
ALT elevation	69	4	14	2	48	0	42	8	66	4	73	9
AST elevation	55	3	9	1	46	0	29	3	40	1	48	3

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

patients demonstrate a five-fold upper limit of normal (ULN) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) activity, which can be improved by dosage adjustment and treatment discontinuation.

In a real-world study of nilotinib, a marked elevation of ALT and AST levels was not common, and the incidence of an increase to more than five-fold the ULN was 1%–4%. Concurrently, the manifestation of jaundice was mainly attributed to indirect bilirubin levels and self-remission, and it was attributed to Gilbert syndrome (GS)[9]. However, in a nilotinib phase I and II study spanning 36 mo and conducted in Japan, 29% of the patients showed elevated bilirubin levels, 24% showed elevated ALT levels, but only 3% showed over five-fold the ULN[10]. Severe liver failure caused by nilotinib treatment is rare. For example, a 34-year-old woman with CML developed progressive jaundice (bilirubin 14.5 mg/dL, ALT 1856 U/L, and ALP 254 U/L) after 8 mo of nilotinib treatment. She was subsequently diagnosed with liver failure and underwent liver transplantation.

COMPARISON OF HEPATOTOXICITY INDUCED BY NILOTINIB AND IMATINIB

Nilotinib is clinically used because of resistance to or side effects of imatinib treatments. In the ENESTnd trial, a phase 3 randomized trial including 846 patients[11], two different doses of nilotinib showed ALT activity rates of over five-fold the ULN at 4% and 9%, respectively, compared with 3% with imatinib. Only two patients showed relatively severe liver dysfunction (data not reported), indicating that the frequency of liver dysfunction due to nilotinib treatment was higher than that of imatinib. During the 5-year follow-up of the same group of patients, the rate of abnormal liver function was 50%–60%, but liver failure was implicated in none of the reported 50 cases of death[12]. A 47-year-old woman with CML presented with liver function deterioration (bilirubin level 20 mg/dL, alanine aminotransferase level 828 U/L prothrombin time 24 s) during imatinib therapy administrated before liver transplantation, and the postoperative treatment with nilotinib did not result in liver damage[13]. In a group of 88 imatinib-resistant CML patients who received nilotinib for 3 years, 14% showed elevated ALT and ALP levels. Interestingly, five patients with imatinib-induced hepatotoxicity did not exhibit any recurrence of nilotinib-induced hepatotoxicity[14].

THE MAIN SYMPTOM OF NILOTINIB-INDUCED HEPATOTOXICITY IS UNCONJUGATED HYPERBILIRUBINEMIA

Elevated levels of bilirubin are a common adverse drug reactions to nilotinib treatment, which occurs in 3%–16% of the patients, but constitutes no evident pathological significance[15]. In a previous study, 119 patients with CML with imatinib resistance were treated with nilotinib; nine patients subsequently showed elevated levels of indirect bilirubin, and three showed elevated ALT levels. None of

the increased parameters influenced the hematological remission effect[16].

In studies on nilotinib-induced hepatotoxicity, hyperbilirubinemia is the most common etiology of hepatotoxicity and is the main reason reducing or discontinuing nilotinib (Table 1). Hyperbilirubinemia is mainly characterized by the presence of unconjugated bilirubin. The initial median time of increase was 18 d, and the duration was approximately 8 d. A spontaneous decrease in bilirubin levels may occur without medication or phototherapy.

In a case reported by Cortes *et al*[17], a 39-year-old man with CML was administered with low-dose (300 mg/d) nilotinib for continuous treatment in the frontline GIMEMA trial, and his bilirubin levels increased gradually, resulting in the development of grade-4 hyperbilirubinemia. Ten days after drug discontinuation, the hyperbilirubinemia was resolved. Moreover, the original dose of nilotinib was continued, and hyperbilirubinemia development was observed along with four recurrences. Bilirubinemia was maintained at grade 2–3, and notably, the patients demonstrated a complete molecular response 1 year after treatment. Therefore, hyperbilirubinemia is considered a benign condition[18].

NILOTINIB-INDUCED HYPERBILIRUBINEMIA IS ASSOCIATED WITH URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE (UGT1A1) ACTIVITY

UGT1A1 is a membrane protein that binds to the endoplasmic reticulum. It is an enzyme that plays a crucial role in phase II biotransformation of several endogenous and exogenous substances[19]. The *UGT1A1* gene is predominantly expressed in the human liver, but also in bile tissue, the large intestine, and the stomach, and it is responsible for bilirubin binding[20]. UGT1A1 is a key enzyme involved in the metabolism of bilirubin. UGT1A1 expression is correlated with UGT1A1 activity, which leads to abnormal synthesis of unconjugated bilirubin in the blood and excretion of conjugated bilirubin *in vitro*[21], resulting in hyperbilirubinemia[22]. Hyperbilirubinemia has been observed in patients treated with TKIs. A correlation between the inhibition of UGT1A1 activity and an increase in bilirubin level was observed in patients treated with TKI. The gene polymorphism of *UGT1A1* is the main reason underlying the function of the *UGT1A1* gene[23].

The promoter region of the *UGT1A1* gene exhibits a certain polymorphism, and its atypical TATA box region contains 5–8 TA repeats. The most common genotypes included six TA repeats[24]. *UGT1A1* expression decreases with increasing TA repeats. A variant of *UGT1A1* containing an untypical TATA box region of the *UGT1A1* * 28 promoter contains seven TA repeats; it is associated with decreased UGT1A1 expression and frequently causes hyperbilirubinemia of unknown etiology[25]. Furthermore, new variants have been reported, including *UGT1A1* *6/*6, *6/*28, and *28/*28[26].

Nilotinib inhibited UGT1A1 activity in a dose-and time-dependent manner. Abumiya *et al*[27] investigated 34 CML patients treated with low and high dosages of nilotinib, and hyperbilirubinemia was more common in patients treated with high dosages and over long periods. The proportion of patients harboring *UGT1A1* *6/*6 and *6/*28 genotypes (poor metabolizers) was also higher[27]. This suggests that variations in the *UGT1A1* gene should be considered when administering nilotinib.

MECHANISM UNDERLYING NILOTINIB-INDUCED HYPERBILIRUBINEMIA

Nilotinib inhibits the activities of cytochrome P450 enzymes CYP3A4, CYP2C8, CYP2C9, CYP2D6, and UGT1A1[28]. Fujita *et al*[29] have proven nilotinib to be an effective non-competitive drug for human UGT1A1 *in vitro*. In this study, SN-38 was used as a substrate, and human liver microsome and recombinant human UGT1A1 were used as enzyme sources to study the inhibitory effect of nilotinib on UGT1A1-catalyzed SN-38 glucuronidation. The results showed that nilotinib exerted a noncompetitive inhibitory effect on SN-38 glucuronization of human liver microsome UGT1A1 and recombinant human UGT1A1 with K (i) values of 0.286 ± 0.0094 and 0.079 ± 0.0029 μm, respectively.

UGT1A1 GENOTYPE DOES NOT AFFECT NILOTINIB EFFICACY AND SAFETY

TKIs are currently used for the treatment of CML. Nilotinib can increase the level of bilirubin by inhibiting UGT1A1 activity[30,31] In imatinib- and dasatinib-treated GS patients, elevated levels of bilirubin may occur; thus, it is not known whether such a condition of hyperbilirubinemia affects the efficacy and safety of nilotinib. We previously reported the case of a 24-year-old CML patient with unconjugated hyperbilirubinemia treated with nilotinib[32]. Reduction and discontinuation of treatment can improve bilirubin levels immediately, but complete cytogenetic response (CCyR) worsens. CCyR can be achieved using a normal dose, but hyperbilirubinemia without ALT and other abnormal enzyme activities can be induced again. After conducting repeated trials four times, a normal dose of nilotinib was administered, and no pathological damage was observed in liver pathology. Although high bilirubin (grade 3–4) levels persisted, the patient achieved continuous CCyR.

In a 16-year retrospective study[33], long-term hematological toxicity and hepatocellular toxicity of imatinib, nilotinib, and dasatinib were observed. The effect of the GS genotype on progression-free survival was evaluated. One hundred and five patients with CML-CP who were consecutively treated with either first- or second-generation TKIs were evaluated. Gilbert's syndrome genotypes were distributed as follows: 17 (16.2%) patients with 7/7, 44 (41.9%) patients with 6/7, and the remaining cases with wild-type genotypes. The results showed that there was no difference in either the major molecular response or complete cytogenetic response observed among the different GS genotypes. Hyperbilirubinemia was observed in 26 patients, and grade 3/4 was observed in 9 patients. However, no true endothelial toxicity was observed in patients who continued to use TKIs.

Therefore, the European LeukemiaNet recommendations state that hyperbilirubinemia caused by TKIs is more common during nilotinib treatment and that this characteristic is the most frequently reported laboratory adverse event, rather than representing a true liver injury. This phenomenon is more common in the UGT1A1-mutated population and should be monitored closely, while dose adjustment has not been suggested[34].

This type of hyperbilirubinemia does not necessitate dose adjustment to achieve improvements, but bilirubin levels may spontaneously decline, a phenomenon which has been recognized by several scholars[18].

CONCLUSION

In conclusion, nilotinib is a commonly used drug in the treatment of CML, during which hyperbilirubinemia is commonly observed, and hyperbilirubinemia with indirect bilirubin elevation is not accompanied by abnormal hepatocyte enzymes. This abnormality is not real drug-induced liver damage, but the variation in UGT1A1 leads to inhibition of UGT1A1 activity, resulting in disorders of the bilirubin metabolism [34]. Therefore, neither dose modification nor discontinuation is recommended for patients with hyperbilirubinemia at more than three-fold the ULN, and the benefits of maintaining the original dose to CML outweigh the disadvantages caused by dose reduction or treatment discontinuation. A different abnormality, mainly accompanied by elevated levels of liver enzymes, should follow the standard of reduction or drug discontinuation at more than three-fold the ULN.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 496-504

DOI: 10.13105/wjma.v9.i6.496

ISSN 2308-3840 (online)

OPINION REVIEW

Preclinical safety, effectiveness evaluation, and screening of functional bacteria for fecal microbiota transplantation based on germ-free animals

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Author contributions: Yang YP, Zhang XY and Cui BT contributed equally to this work; Wei H conceptualized the review; Yang YP drafted the manuscript; Zhang XY and Cui BT revised the manuscript; Zhang H, Tan X, Wu ZF, Cheng W and Zhu X reviewed and compiled the data and drafted the manuscript; Zhang FM and Qin HL made critical comments on the manuscript; all authors have read and edited the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests for this article.

Supported by National Natural Science Foundation of China, No. 8177020321; and the Emerging Cutting-Edge Technology Joint Research Projects of Shanghai, No. SHDC12019114.

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Abstract

Fecal microbiota transplantation (FMT), also known as fecal bacterial therapy, is a treatment option that can quickly reconstruct the normal composition of intestinal microbes, and it has a good therapeutic effect on *Clostridium difficile* infection, as well as on other microecological disorders. However, the causal mechanism of FMT efficacy remains to be clarified, its safety is a major problem, and the standardization and acceptability of FMT need to be improved. This review summarizes its current research status and potential research areas that need to be strengthened, and proposes to clarify the safety of FMT and the causal relationship between FMT and therapeutic effectiveness based on germ-free animals. Meanwhile, the research system is combined with multiomics technology to screen the effective bacteria in FMT, and develop standard, safe, effective and

Country/Territory of origin: China

Specialty type: Microbiology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): E

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Received: April 8, 2021
Peer-review started: April 8, 2021
First decision: October 3, 2021
Revised: October 14, 2021
Accepted: December 24, 2021
Article in press: December 24, 2021
Published online: December 28, 2021

P-Reviewer: Leardini D, Link A
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Liu M

controllable flora of FMT.

Key Words: Fecal microbiota transplantation; Effectiveness; Safety; Germ-fee animals; Functional bacteria

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Core Tip: Gut microbiota plays an important role in the development and treatment of many diseases, especially gastrointestinal disorders. Fecal microbiota transplantation (FMT) has potential application in treating various diseases. However, the mechanism of FMT is unclear, its safety needs to be improved, and it is not standardized due to a variety of reasons. Therefore, in this review, we analyze the current dilemma of FMT and propose the importance of establishing a germ-free animal evaluation system, with an aim to help establish standard, safe, effective, controllable, and product-oriented formula flora of FMT.

Citation: Yang YP, Zhang XY, Cui BT, Zhang H, Wu ZF, Tan X, Cheng W, Zhu X, Zhang FM, Qin HL, Wei H. Preclinical safety, effectiveness evaluation, and screening of functional bacteria for fecal microbiota transplantation based on germ-free animals. *World J Meta-Anal* 2021; 9(6): 496-504
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/496.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.496

INTRODUCTION

Fecal microbiota transplantation (FMT) refers to the transplantation of healthy human intestinal flora into the intestinal tract of patients to treat diseases by reconstructing the normal composition of intestinal microbes[1]. The earliest record of treating diseases with human feces was written in the book "Zhou Hou Bei Ji Fang" by Ge Hong in the fourth century AD, which was used to treat severe food poisoning[2]. In modern medicine, FMT is a treatment option that can quickly reconstruct the normal composition of intestinal microbes[3]. It is recommended by clinical guidelines for treatment of recurrent *Clostridium difficile* (*C. difficile*) infection (CDI)[4-6] and acute graft-versus-host reaction (GVHD)[7]. In addition, it has been found that FMT has potential therapeutic value for inflammatory bowel disease (IBD), irritable bowel syndrome, autism, metabolic syndrome, antibiotic-associated diarrhea, hepatic encephalopathy, and other related diseases[1,8,9], even systemic diseases associated with intestinal microbiota, such as obesity[10]. Although FMT has been shown to have potential in treating various diseases, its therapeutic mechanism remains unclear[11]. Like other treatments, FMT needs to be scientifically evaluated for its advantages and disadvantages, and its safety needs to be enhanced[12]. The degree of FMT standardization is also low due to different donors for this technique[11] and separate guidelines in different regions[9]. Therefore, in this review, we discuss the use of germ-free animal systems to understand the mechanism of FMT efficacy and screen the functional strains of FMT, to discover the functional bacteria that play a role in FMT, and develop a safe, controllable, standard, and effective combination of functional bacteria.

CAUSAL RELATIONSHIP AND MECHANISM OF FMT EFFICACY NEEDS TO BE CLARIFIED

CDI is an important risk factor for exacerbation and death in hospitalized patients, with recurrent infection occurring in up to 30% of patients receiving conventional treatment, suggesting that the mechanism of antibiotic treatment for CDI is the direct killing of *C. difficile*, but it may not be eradicated. FMT is a new approach to solve this complex problem. In the treatment of recurrent CDI, the effective rate of FMT is nearly 90%[13]. Different from the direct killing effect of antibiotics, the recovery of intestinal

flora diversity is considered as the main mechanism of FMT in treating CDI[14]. FMT has been proved to be effective in treating an increasing number of diseases, and its clinical application continues to grow. For example, the efficacy of FMT in the treatment of IBD has also been confirmed. By including 75 patients with ulcerative colitis (UC), Costello *et al*[15] demonstrated that FMT was significant in relieving UC, with nine of 38 patients (24%) in the FMT group having remission and two of 37 patients (5%) in the placebo group having remission at the end of treatment.

FMT has great clinical potential, and it is a type of donor-specific treatment, with heterogeneous efficacy for different pathological conditions. At present, the whole spectrum of bacteria and randomness of donors are still used for clinical treatment [16], and there is no universal super feces[17]; it is therefore necessary to clarify the mechanism of FMT to better exert its effects in diseases other than CDI. According to existing research reports, FMT works through direct or indirect approaches. Direct approaches include the introduction of bacteriophages, bacteriocins, and metabolites (short-chain fatty acids and bile acids), and nutritional competition between normal flora and *C. difficile*. Indirect approaches include participation in the metabolism of short-chain fatty acids and bile acids, thus participating in the regulation of immune and mechanical barriers to exert efficacy[9,18]. It has been found that human FMT compensates for the microbial imbalance in the feces of colitis rats by restoring diversity and increasing the relative abundance of health-related microbes[19]. FMT also regulates the microbial composition of the colon, which leads to recovery of colon length, and significantly reduces the severity of epithelial injury and disease[19]. Natividad *et al*[20] found that ecobiotherapy rich in Firmicutes can reduce the susceptibility to colitis in a humanized gnotobiotic mouse model, and downregulate colonic inflammation and TH17 pathways in mice; it is clear that the mechanism of FMT against colitis may be the enriched Firmicutes[20].

SAFETY OF FMT NEEDS TO BE EVALUATED

Currently, FMT has been applied to clinical treatment of various diseases. However, as an emerging clinical treatment, the short- and long-term safety issues of FMT have become the focus of attention, requiring further clinical and laboratory data to confirm. China and the United States have established the China Microflora Platform (www.cmts.com)[21] and the United States FMT National registry system (FMT National Registry System)[22,23], respectively, for the follow-up and registration of all patients receiving FMT for up to 10 years to assess its long-term safety. In the systematic review reported by Zhang Faming's research group from 2000 to 2020, FMT-related adverse events were divided into transplantation-related and microecologically related adverse events for the first time, and mucosal barrier injury-associated microbiota-related adverse events were defined. The total incidence of FMT-related adverse events was 19%, with diarrhea, abdominal discomfort, pain, and spasm being the most common, and the incidence of FMT-related serious adverse events was 1.4%. The incidence of microecologically related serious adverse events was 0.99%, and all serious adverse events occurred in patients with impaired intestinal mucosal barrier function[24].

FMT is associated with a risk of infection and needs to be taken seriously. As with all treatments, the advantages and disadvantages need to be assessed[12]. For example, FMT has been used as a potential treatment option for Crohn's disease (CD), but there is still a lack of evidence for safety based on the large number of CD samples undergoing FMT. The study by Wang *et al*[25] showed that 184 FMT frequency tests were performed in 139 patients receiving FMT, and 13.6% of mild adverse events including fever, abdominal pain, flatulence, hematochezia, vomiting, abdominal distension, and increased frequency of herpes zoster occurred within 1 mo after FMT. In two separate clinical trials, after FMT, two patients developed extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* bacteremia; both of which were related to the same fecal donor, and one of the patients died. Therefore, donor screening should be enhanced to limit microbial transmission that could lead to adverse infectious events to determine the benefits and risks of FMT in different patient populations[26]. A multicenter retrospective study by Nicholson *et al* confirmed that the treatment of CDI in pediatric patients was safe and effective, but 4.7% of patients still had serious adverse events during a follow-up period of 3 mo[27]. Ianiro *et al*[28] studied the safety of FMT for recurrent CDI in 290 patients; 109 were treated with FMT and 181 with antibiotics. After 90-d treatment, 40 patients in the antibiotic group had bloodstream infection (BSI). The analysis of matched patients showed that compared

with antibiotic treatment, the risk of BSI in the FMT group decreased by 23%, but there were still five cases of BSI in the FMT group. Bilinski *et al*[29] published a case report in which the patient developed severe diarrhea and norovirus infection after FMT, accompanied by grade III nausea, weight loss, and rising blood eosinophilia. DeFilipp *et al*[30] hypothesized that FMT may restore intestinal microbiome diversity after allogeneic hematopoietic cell transplantation (allo-HCT). In this open-label single-group pilot study, 13 patients received FMT capsules. Some patients developed grade 3-4 acute gastrointestinal tract GVHD after FMT, and one of these patients developed *Klebsiella pneumoniae* bacteremia and sepsis, followed by multiple organ failure. There was one case of *C. difficile* colitis observed after FMT and six patients developed moderate-severe chronic GVHD[30]. Take into account this, the FDA issued a warning to researchers that fecal screening in FMT studies should be expanded to include specific resistant strains[31] and opportunistic pathogens should also be considered. In addition, when improving the safety of FMT to reduce the risk of systemic infection (such as sepsis caused by FMT spreading drug-resistant pathogens), besides screening for pathogens in the donor fecal bacteria, the composition and structure of the flora should also be considered, such as flora diversity, key bacterial species (such as short-chain fatty acid-producing bacteria), and the ratio of obligate and facultative anaerobic bacteria, which can improve the safety of FMT[31]. At present, there is insufficient evidence to prove that FMT is used to treat diseases other than CDI, and long-term safety still needs to be evaluated[32].

At present, FMT has not been approved as a new drug, which is primarily due to the lack of preclinical safety studies. Experimental animal research can clarify the relationship between safety and bacterial solution treatment methods, dosage, and other factors, and provide scientific data in support of the safety of FMT in clinical application. Since 2014, Zhang Faming's team has begun to use the automatic fecal isolation system (genFMTer) to ultrafilter, purify, and centrifugally wash the fecal microbiota[33,34], and select different routes for transplantation into patients, which is defined as washed microbiota transplantation (WMT)[4,35]. Preclinical studies have confirmed the safety of WMT after automatic machine isolation in experimental animals, and its clinical application can significantly reduce FMT-related adverse events without affecting the efficacy of patients[4,25,36].

STANDARDIZATION AND ACCEPTABILITY OF FMT SHOULD BE IMPROVED

The material of FMT is variable, which is caused by biological variation and sample handling. Unlike drugs, FMT products are more personalized due to different donors [11]. Human feces (microorganisms and chemical components) vary greatly from person to person. Even among healthy individuals, the diversity and abundance of microorganisms in each individual vary widely, and there is a strong niche specificity within and between individuals, and many diseases are linked to specific microorganisms and chemicals in feces[37]. Therefore, during the process of FMT, different donors and improper preparation of samples may lead to differences or variations in these microorganisms, affecting the therapeutic effect and even causing adverse events. In addition, the choice of treatment for FMT varies from region to region, resulting in low standardization of FMT[9].

However, there is no clear and universal screening standard for fecal donors. The reported screening methods are strict, and usually < 20% of volunteers meet the screening standards of blood, feces, and urine[38]. Therefore, it is obviously necessary to develop FMT substitutes for patients with microecological disorders, including bacteria, fungi, protozoa, viruses, cytokines, and metabolites, and it is necessary to determine which of them are beneficial factors, which will establish a link between the disease and beneficial microorganisms of therapeutic value[11]. In addition, the development of targeted probiotic microorganisms or combinations can be considered, and alternatives including bacteriophages should also be considered[12], which may be an important research direction.

Recent investigations of doctors, medical students, donors, and patients have shown that FMT is less acceptable compared with traditional treatment methods, especially fecal bacterial suspension prepared by the rough method[4,39,40]. Zhang *et al*[4] found that the bacteria prepared by standardized formula have greater acceptance with the same efficacy, while improving the activity of the flora, which meets the standards of modern medical aesthetics. Therefore, it is necessary to improve the standardized preparation technology of fecal suspension to improve the acceptability of FMT, and

the screening of effective bacteria or metabolites should be considered to improve the standardization and acceptability. In 2019, China's first national consensus opinion on the methodology for WMT was confirmed and published, aiming to regulate the processes such as donor screening, fecal microbiota preparation, storage and transportation, selection of transplantation route, and safety monitoring and management of FMT, so as to improve the safety of FMT and ensure its clinical efficacy[35].

Therefore, screening of the functional strain is prepared under the condition of clarifying the mechanism of action of the FMT effective strain. At the same time, FMT operation procedures are improved to reach a consensus, which makes the FMT strain preparation easier to control the production, safer, more acceptable, and more standardized.

PRECLINICAL SAFETY, EFFECTIVENESS EVALUATION, AND EFFECTIVE MICROBIAL SCREENING OF FMT BASED ON GERM-FREE ANIMAL SYSTEMS

Germ-free animals refer to animals without microorganisms living in or on them. These animals have become an irreplaceable research tool to study the relationship between a single strain or multiple strains and the host, and also an indispensable model for studying functional microorganisms in humans and animals[41,42]. Germ-free animal disease models can be used to evaluate the effectiveness of FMT, to study the causal relationship between microbiota transplantation and therapeutic effect, and clarify the mechanism of action between FMT and disease in the absence of host microbial interference. In addition, germ-free animals can be evaluated for FMT safety and provide preclinical scientific data for FMT by evaluating cardiac, liver, and renal toxicity, immune disturbance, impact on behavior, and the risk of local and translocation infection. The use of germ-free immunodeficient animals can correspond to patients with low clinical immune function, and the humanized flora can be transplanted to germ-free immunodeficient animals for FMT safety test, to enhance the sensitivity of FMT activity and ensure the preclinical safety of FMT to treat immunodeficient individuals. For example, two immunocompromised patients developed ESBL-producing *E. coli* infection after FMT and one of them died[26]. However, safety assessment with germ-free immunodeficient animals in advance may avoid this adverse event.

Papanicolas *et al*[31] reviewed that the protection of symbiotic bacteria and the reduction of pathogenic bacteria in feces could improve the safety of FMT, and the change in fecal flora might reduce the risk of sepsis. Technology based on germ-free animals and multiomics provides the possibility to modify the fecal flora. Based on the clear causal relationship between FMT and germ-free animal models of human diseases, a function-oriented system for screening and evaluating transplanted flora could be established. By using metagenomics and cultureomics, effective target bacteria of FMT are obtained, and the formula flora with a clear function can be formed, so that the functional bacteria of FMT can be visible, accessible, and available.

The use of germ-free animals to standardize the preclinical safety evaluation of FMT, establish an effective evaluation system that can clarify the causal relationship and mechanism of the efficacy of FMT, and combine multiomics technology to screen the effective bacteria can improve the safety, standardization, controllability, and acceptance of FMT. Therefore, we propose a safety and effectiveness research system of FMT based on germ-free animals, and the screening of FMT efficient bacteria (Figure 1): (1) The causal relationship between therapeutic effect and flora transplantation is validated. For patients who have been effectively treated with FMT, the flora before and after FMT was transplanted into the corresponding germ-free disease model mice to verify the causal mechanism of FMT efficacy. We can use the standard germ-free disease model for FMT effectiveness evaluation, to ensure the scientific validity and versatility of FMT effectiveness data, clarify the causal relationship between flora transplantation and therapeutic effect, and clarify the mechanism of FMT; (2) The humanized flora model is constructed based on germ-free animals to evaluate the safety of FMT. The preclinical safety evaluation of FMT is conducted in strict accordance with the international classics and standard preclinical safety evaluation specifications of new drugs, medical devices, and medical technology. In addition, germ-free immunodeficient animals were used to evaluate the safety of FMT; cardiac, liver, and renal toxicity of FMT; interference with the immune

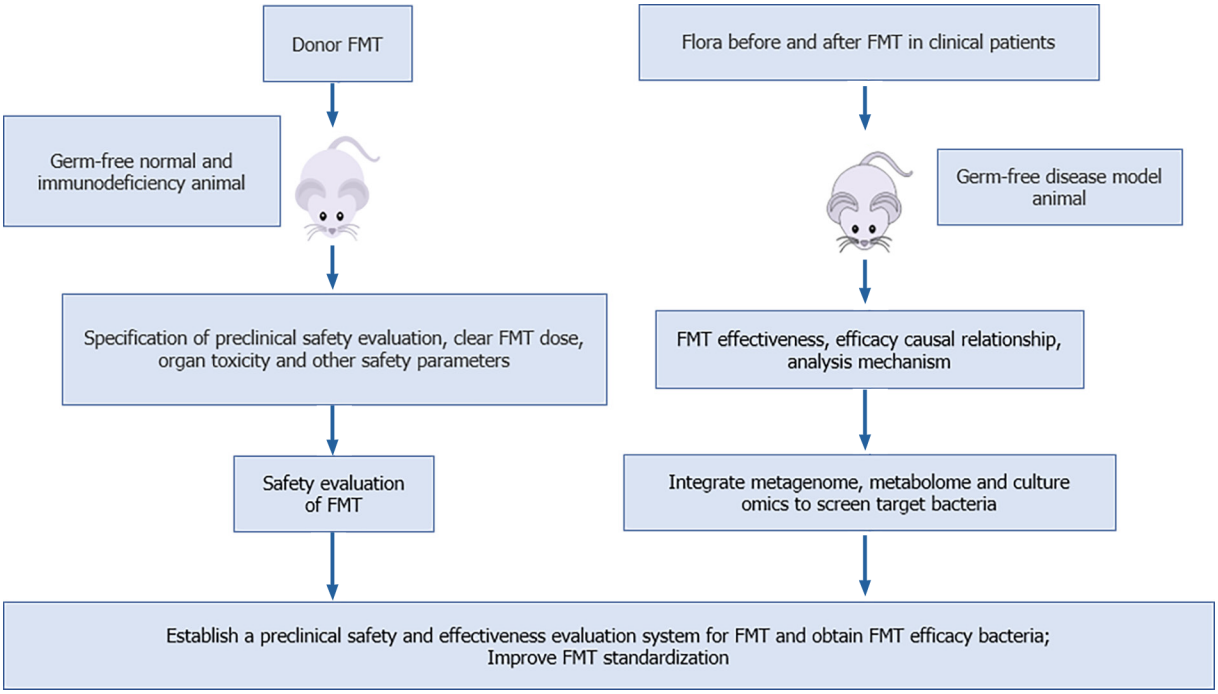


Figure 1 Preclinical safety, effectiveness evaluation, and effective bacterial screening of fecal microbiota transplantation based on germ-free animals. An FMT safety evaluation system is established based on germ-free normal and immunodeficient animals. The causal mechanism for the efficacy of fecal microbiota transplantation (FMT) is verified based on germ-free disease animal models, to clarify the effectiveness of FMT, and multiomics technology is used to screen the effective bacteria of FMT, and to improve the standardization of FMT. FMT: Fecal microbiota transplantation.

system; behavioral effects of FMT; and the risk of local and translocation infections. Germ-free immunodeficient animals are also used to enhance the sensitivity of FMT, and better identify the route and dose of FMT, and other indicators to ensure its safety; and (3) At present, there are insufficient studies to obtain the effective flora of FMT. Therefore, after clarifying the causal relationship of FMT in step one, we can combine with metagenomics and cultureomics techniques to establish a function-oriented system for screening, obtaining, and evaluating flora, and finally, the effective target bacteria of FMT can be obtained and functional formula flora can be formed. The efficacy of the formulated bacteria can be verified again by using germ-free animal models of human diseases, so as to achieve standardization of FMT, improve the acceptance level, and reduce infection risk.

CONCLUSION

Gut microbiota plays a major role in the development and treatment of many diseases, especially gastrointestinal disorders. FMT is a therapeutic option that can rapidly reconstruct the normal composition of intestinal microorganisms. FMT has potential to treat various diseases, such as CDI, IBD, constipation, and cancer. However, the therapeutic mechanism of FMT is unclear, its safety needs to be improved, and it is not standardized due to a variety of reasons. Germ-free animals, as the most appropriate models for studying the interaction between microorganisms and hosts, are applicable research tools for studying the mechanism of action of FMT, screening the functional bacteria of FMT, evaluating the safety of FMT, and improving standardization of FMT. Therefore, evaluation of the safety and effectiveness of FMT animal experiments based on germ-free animals will be set up. By using a function-oriented *in vivo* experimental system, combined with clinical analysis and culturomics technology, the functional strains of FMT for microbial diseases can be obtained and verified. And the standard, safe, effective, controllable, and product-oriented flora of FMT can be developed.

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World J Meta-Anal 2021 December 28; 9(6): 505-521

DOI: 10.13105/wjma.v9.i6.505

ISSN 2308-3840 (online)

REVIEW

Gastrointestinal tumors and infectious agents: A wide field to explore

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Conflict-of-interest statement: The authors declare no conflict of interest regarding the elaboration

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Abstract

Infection is currently one of the main contributors to carcinogenesis. In fact, the International Agency for Research on Cancer has categorized eleven biological agents as group I carcinogens. It is estimated that around 16% of the 12.7 million new cancers diagnosed in 2008 were attributable to infectious agents. Although underdeveloped regions carry the highest incidence rates, about 7.4% of infection-related cancer cases occur in developed areas. Physicians are increasingly aware of the potential carcinogenic role of common virus like the Human Papilloma virus in cervical cancer, or the hepatitis B and C viruses in hepatocarcinoma. However, the carcinogenic role of several other infectious agents is less recognized. Given that gastrointestinal malignancies carry an overall poor prognosis, a better understanding of the carcinogenic mechanisms triggered by infectious agents is key to decrease the rate of cancer related deaths. Preventive measures directed to such infections would ideally impact survival. In this paper

of this paper.

Country/Territory of origin: Spain

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

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Received: May 1, 2021
Peer-review started: May 1, 2021
First decision: July 4, 2021
Revised: August 26, 2021
Accepted: December 23, 2021
Article in press: December 23, 2021
Published online: December 28, 2021

P-Reviewer: Huan C, Zheng ZX
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

we review the main pathogenic mechanisms related to the development of gastrointestinal malignancies induced by infectious microorganisms and other pathogens which are currently under investigation.

Key Words: Gastrointestinal tumors; Infectious agents; Bacteria; Virus; Prevention

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Core Tip: Except for pathogens with well-known carcinogenic potential, such as Human Papilloma virus or Hepatitis C virus, physicians are usually unaware of the relationship among other infectious agents and tumors. The identification and subsequent eradication of these pathogens might help to prevent the development of a large number of tumors. Gastrointestinal malignancies are usually related to a very poor outcome. Therefore, detection of carcinogenic pathogens in this population might help to increase overall survival. Screening strategies and further research is required to face with these preventable diseases.

Citation: López-Gómez M, García de Santiago B, Delgado-López PD, Malmierca E, González-Olmedo J, Gómez-Raposo C, Sandoval C, Ruiz-Seco P, Escribano N, Gómez-Cerezo JF, Casado E. Gastrointestinal tumors and infectious agents: A wide field to explore. *World J Meta-Anal* 2021; 9(6): 505-521
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/505.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.505

INTRODUCTION

During the last decades the causal relation between several pathogens and the onset of cancer has been firmly established[1]. However, the relationship between pathogens and cancer has been subject of research since the end of the XIX century. Rous[2,3] (1879-1970) discovered that sarcomas affecting domestic fowls could be transferred to other fowls through a viral vector, later known as Rous sarcoma virus (RSV). This finding was awarded with the Nobel Prize in 1966 and demonstrated for the first time in history that a malignant tumor could be induced by an infectious agent. In fact, the RSV was considered the first oncogenic retrovirus. Subsequently, other tumor-inducing viruses affecting animals were identified[4-6]. In 1964, the first oncogenic virus affecting humans, the Epstein Barr virus, was identified[7].

At present, a great effort is being done in order to elucidate the underlying connection between infectious agents and cancer. In 2018, an estimated 2.2 million infection-attributable cancer cases were diagnosed worldwide, corresponding to an age-standardized incidence rate (ASIR) of 25.0 per 100.000 person-years[8]. Currently, 11 infectious agents have been classified as Group 1 Carcinogens by the International Agency for Research on Cancer (IARC)[1]. *Helicobacter pylori* (*H. pylori*) (810.000 cases, ASIR 8.7), Human Papilloma virus (690.000, 8.0), Hepatitis B virus (360.000, 4.1) and hepatitis C virus (HCV) (160.000, 1.7) comprise the primary causes of infectious-related tumors, accounting for more than 90% of infection-related cancers worldwide[9].

VIRAL INFECTIONS

Viral chronic infection can promote cancer *via* three different pathogenic mechanisms:

(1) Induction of chronic inflammation provoked by the immune response to a persistent infection; HCV-related liver cancer is a paradigmatic example, in which persistence of viral replication within the liver parenchyma maintains a state of local chronic inflammation involved in cancer development[10]; (2) Virus-induced genome transformation secondary to viral persistence in infected cells; This mechanism is typically found in Epstein-Barr virus (EBV) associated Burkitt's lymphoma. EBV replicating in the oral epithelium can transform resting B lymphocytes into proliferating lymphoblastoid cell lines involved in oncogenesis[11]; and (3) Promotion of chronic suppression of host immune response. Patients infected with the human

immunodeficiency virus can be deeply immune-suppressed. These individuals exhibit an increased predisposition to develop infection-driven tumors as the mechanisms of immunosurveillance are disrupted by the viral replication[12].

Although these mechanisms may occur simultaneously[13], some other yet unknown need also be involved, since many other pathogens inducing similar immune alterations do not seem to induce cancer. It is widely recognized that viruses can cause cellular malignant transformation by inducing genetic instability. In fact, viruses are able to fuse their genome with that of the host resulting in activation of multiple oncogenes and intracellular signaling pathways leading to cellular proliferation, inflammation and immune dysregulation[14,15].

BACTERIAL INFECTIONS

Recent studies have shown that certain infectious agents like molds, helminths and bacteria, capable of interacting with mammalian host cells, can induce cancer, even in the absence of genomic alteration[16]. Unlike the well-established relation between viruses and carcinogenesis, the oncogenic role of bacterial infection remains controversial[17]. The molecular mechanisms by which bacteria might promote carcinogenesis are currently under research[10], and both bacterial and host factors seem to be involved. Bacterial cell-surface components, toxins and effector proteins can interact with host cells by activating essential signaling pathways involved in cancer formation [16].

Role of bacterial surface antigens in carcinogenesis

The bacterial surface exhibits several antigens that interact with the host and activate both innate and adaptative immune responses, allowing them to escape the host immune system. For example, gram-negative bacteria can coat their outer surface with a polysaccharide capsule, thus preventing the activation of the Complement cascade and the phagocytic process[18]. Other bacteria are able to modify some surface molecules, like lypo-polysaccharides, flagella and peptidoglycans, to avoid immune recognition[19]. Additionally, some other bacteria can express a variety of surface proteins that facilitate attachment to host cells and subsequent internalization[20]. These strategies aim to improve bacterial survival by eluding the immune response. However, definitive cellular malignant transformation needs to be induced by specific intracellular signaling cascade activation driven by certain bacterial molecules. For example, *H. pylori* can directly activate the RAS/mitogen activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway resulting in malignant transformation *via* the CagL protein (an adhesin molecule) that binds to the beta5-integrin expressed by host cells[21]; *H. pylori* also expresses the OipA protein that binds to the EGFR host receptor resulting in AKT and beta-catenin signaling activation, which also promotes carcinogenesis[22].

Immune cell elimination

To ensure survival among host cells, pathogenic bacteria have developed several strategies to attack the immune system. For example, they can secrete cytolytic toxins through their outer membranes that can affect the endosomal system. Bacterial toxins may induce carcinogenesis altering the host cell environment by inducing genome instability, promoting resistance to cell death signaling, and enhancing proliferative signaling[23].

Induction of genome instability: Bacterial toxins are capable of inducing host cell damage in the DNA double helix. Cytolethal Distending Toxin, Collibactin, Shiga toxin and endonucleases are some examples[24]. DNA damage causes immune host cells to arrest the cell cycle at the G1-S or G1-M stages.

Resistance to cell death signaling and induction of proliferative signaling: Toxins produced by some pathogens, like *Bacteroides fragilis*, can bind to intestinal epithelial cell receptors and induce cellular proliferation and differentiation. *Bacteroides fragilis* can silence the tumor suppressor protein E-Cadherin, resulting in the activation of beta-catenin/Wnt and nuclear factor kbeta (NF-kB) signaling pathway[25].

Host cells transformation secondary to bacterial proteins

To induce malignant transformation, bacteria must ensure a persistent infection within the cells of the new organism. They need to be internalized, must control their own

Table 1 Pathogens related to gastrointestinal malignancies

Ref.	Primary tumor	Pathogen	Molecular mechanisms
von Knebel Doeberitz *et al*[30], Syrjänen *et al*[31], Liyanage *et al*[36]	Esophageal	HPV	Oncoproteins E6 and E7
Wu *et al*[41], Zhang *et al*[42], Wang *et al*[43]		EBV	Oncoproteins EBER and LMP-1
Yang *et al*[44], Tan *et al*[45], Han *et al*[46]		Herpes Simplex and CMV	Unknown
Xu *et al*[56], Mueller *et al*[57]	Gastric	Helicobacter pylori	Cag A *H. pylori* protein
Rassow *et al*[58], Jain *et al*[59]			VacA
Lamb and Chen[61], Brandt *et al*[62]			Interleukins, NFbeta, p53
Haghjoo and Galán[73]	Gallblader	Salmonella typhi	Cytolethal Distending Toxin
Belzer *et al*[75], Maurer *et al*[76]		*Helicobacter* spp.	Urease production
Lax and Thomas[10]		Escherichia coli	Cytotoxic necrotizing factor I
Sripa and Pairojkul[78], Kurathong *et al*[79]		Opisthorchis vierrini and Clonorchis sinensis	Poorly known
Lau *et al*[88], Minami *et al*[89]	Liver	Hepatitis B virus	HBx protein
Raimondi *et al*[92], Akuta *et al*[93]		Hepatitis C virus	HCV core protein
Tahara *et al*[97], Abed *et al*[98]	Colon	Fusobacterium nucleatum	Fap2 protein
Choi *et al*[110], Roxas *et al*[111]		Escherichia coli	CEACAM6, MIC-1
Arumugam *et al*[112], Sears *et al*[113]		*Bacteroides fragilis*	BFT
Del Bel Belluz *et al*[116], Kang *et al*[117]		Salmonella enterica	Typhoid toxin, AvrA effector protein
Palefsky[125], Dyson *et al*[127]	Anal cancer	HPV	E6, E7, p53

HPV: Human Papilloma virus; EBV: Epstein Barr virus; CMV: Citomegalovirus; VacA: Vacuolating cytotoxin; BFT: *Bacteroides Fragilis* toxin.

growth and facilitate their release. Usual non-pathogenic bacteria are typically phagocyted in the phagosome, and then merged with the lysosome and turned into a phagolysosome, where they are finally destroyed. However, some bacteria have developed a number of mechanisms that avoid the formation of the phagolysosome, allowing them to freely enter the cytosol[26]; some bacteria, for example, secrete proteins that induce pore formation in the organelle[27]; while others have developed different mechanisms to avoid destruction inside the phagolysosome[28].

The ultimate explanation by which bacteria promote cancer and whether they obtain any survival advantage remains unknown. This is an interesting and fast-growing research field, in which prevention measures directed to infection may impact overall cancer development. The oncogenic role of various infectious agents regarding gastrointestinal tract malignancies are now described. Since these tumors carry relevant mortality rates, screening programs directed to identify such pathogens may be beneficial.

ESOPHAGEAL CANCER

The incidence of esophageal tumors differs substantially across countries. The highest rates are found in Southern and Eastern Africa and Eastern Asia. Although the histopathological squamous variant has been associated to several viral subtypes, the pathogenic role of viruses in the development of adenocarcinoma is yet unclear.

Human papilloma virus

HPV family viruses are non-enveloped DNA tumor viruses transmitted by sexual contact. More than 100 subtypes of HPV have been described, of which at least 14 carry a significantly increased risk of tumor development [29]. HPV infects epithelial cells and is able to integrate itself inside the host genome. Some oncoproteins

produced by the virus (mainly E6 and E7) alter tumor suppressor pathways and promote malignant proliferation[30]. In 1982, Syrjänen *et al*[31] observed the typical morphological lesions present in HPV related-condylomas in both benign and cancerous esophageal tissues. These findings supported the hypothesis that HPV was involved in the pathogenesis of esophageal malignancies. Subsequent immunohistochemical studies demonstrated that HPV structural proteins were present in malignant lesions of patients from different continents[32]. In fact, the oncogenic potential role of HPV in the development of squamous esophageal carcinoma has been the subject of several metanalysis[33-35]. The first metanalysis of case-control studies investigating the role of HPV in esophageal tumors was conducted in 2013 by Liyanage *et al*[36]. They gathered 21 studies, including 1223 patients and 1415 controls. The authors calculated a pooled OR for HPV and squamous esophageal tumors of 3.04 [95% confidence interval (CI): 2.20 to 4.20]. Noticeably, countries with a low to medium esophageal cancer incidence showed a stronger relationship (OR 4.65, 95%CI: 2.47 to 8.76) than regions with higher incidence (OR 2.65, 95%CI: 1.80 to 3.91). The authors concluded that HPV infection was associated with a 3-fold risk of squamous esophageal cancer and made an urgent call to the IARC to promote the use of vaccines against HPV in high-risk populations.

In 2016, Wang *et al*[37] published a systematic review and meta-analysis on the association between HPV 16 and 18 subtypes and esophageal cancer worldwide. They included 32 studies and showed that HPV infection rate in the tumoral cohort was 46.5% *vs* 26.2% in controls (OR = 1.62; 95%CI: 1.33–1.98), providing strong epidemiologic evidence supporting the association between HPV infection and esophageal tumors[37].

EBV

EBV is an oncogenic virus that belongs to the gamma-herpes virus family. It is a widespread pathogen carried by 95% of the population worldwide. Fortunately, the majority of EBV infections are subclinical[38]. Although, the EBV typically infects B lymphocytes, recent research has shown that it can also infect epithelial cells, and suggests a potential association with esophageal carcinoma, yet to be confirmed. Jenkins *et al*[39] detected for the first time EBV in esophageal tumors, however, only in 5 out of 60 tumors. Awerkiew *et at*[40] demonstrated the presence of EBV-DNA in 35% of squamous cell carcinomas and 36% of adenocarcinomas, using nested polymerase chain reaction (PCR) diagnostic techniques.

In areas with higher incidence of esophageal carcinoma, the association between EBV and esophageal cancer has been investigated. Wu *et al*[41] studied the coinfection of EBV and Herpes Simplex virus (HSV) and detected, by in situ hybridization and immunohistochemistry, the EBER and LMP-1 proteins in 6.1% of carcinoma specimens, preferentially among poorly differentiated squamous cell carcinomas and undifferentiated carcinomas with intense lymphoid infiltration. Other authors have contributed to elucidate the role of EBV in the carcinogenesis of esophageal carcinoma [42,43]. In summary, EBV infection has been related to the etiology of some variants of esophageal carcinoma, at least in countries with increased prevalence. However, more studies are needed to establish the exact pathogenic role of EBV in esophageal carcinogenesis.

Herpes simplex and Citomegalovirus

HSV-1 infection occurs mainly in the mouth and has been related to oral cancer[44]. Citomegalovirus (CMV), a common human pathogen has been associated with cervical and non-melanoma skin cancer[45,46]. Zhang *et al*[42] reported an increased predisposition of esophageal carcinoma in patients infected with HSV-1 (OR 10.3 with 95%CI: 3.331.6). HSV infection is primarily related to esophagitis, which usually precedes esophageal carcinoma. However, no CMV was detected in any of the samples[42]. Wu *et at*[41] found HSV DNA and HSVI, II protein expression in 52 (31.7%) of the 164 tumors analyzed supporting the relationship between HSV and esophageal carcinoma cell differentiation with lymphocyte infiltration in the tumoral stroma. However, other authors have failed to demonstrate the relation between HSV/CMV and esophageal carcinoma, therefore leaving the question open[47].

GASTRIC CANCER

H. pylori

Gastric cancer can be generally classified in cardia (upper stomach) and non-cardia (lower stomach) subtypes. These entities differ in terms of risk factors, carcinogenesis and epidemiologic patterns. Chronic *H. pylori* infection is considered the principal cause of non-cardia gastric cancer, being nearly all cases attributed to this bacterium [48,49]. The worldwide prevalence of *H. pylori* infection is extraordinarily high, affecting around 50% of population[50], and its geographic variability correlates with the incidence of gastric cancer. Yet, less than 5% of infected persons will develop cancer, likely because of differences in bacterial and host genetics, age of infection and environmental factors[51]. In 1994, the World Health Organization categorized *H. pylori* as a Carcinogen type I for gastric cancer on the basis of observational studies reported from the International Agency for Research in Cancer[52].

H. pylori infection has been associated with an increased risk of all variants of adenocarcinoma, whether diffuse or intestinal, and whether located in the body of the stomach or in the antrum; actually, it is related to tumors distal to the cardia. Conversely, tumors growing in the esophagogastric junction, usually arising from the altered mucosa in Barrett's esophagus, have not been linked to *H. pylori* infection[53].

H. pylori survives in the gastric acid microenvironment, being able to damage the mucosa. It induces a chronic inflammatory response that results in chronic gastritis and peptic ulceration. Development of gastric cancer is one of the long-term consequences of *H. pylori* infection[54]. Chronic *H. pylori* infection has been also related to gastric MALT (Mucosa associated Lymphoma), which is an extra-node lymphoma variant consisting of a morphologically heterogenous small B cells neoplasm[55].

H. pylori is able to dysregulate host signaling pathways and to promote oncogenesis by two main mechanisms: Citotoxin-associated gene A (CagA) and its pathogenic island (Cag PAI); and vacuolating cytotoxin A (VacA). CagA *H. pylori*-protein is encoded by one of the genes located in the CagPAI island. CagA binds to the ectodomain of alfa5beta1 integrin allowing its internalization. Once it has been translocated, CagA binds to the inner surface of the cell membrane and undergoes tyrosine phosphorylation. Both the phosphorylated CagA and the unphosphorylated CagA can interact with a number of host proteins to activate downstream signaling pathways such as the RAS/MEK/ERK pathway, the NF-kB pathway, and the beta-catenin pathway. Such pathways activation enhances the proliferation of gastric epithelial cells[56,57].

The VacA is secreted by *H. pylori* and has a variety of biological functions. It binds to host cells and is internalized creating vacuoles (large vesicles) inside the host cells. This *vacuolization* process leads to the activation of endosomes and early lysosomes. Additionally, VacA can also be transferred to the mitochondria where it alters the membrane barrier that releases cytochrome c which finally activates the pro-apoptotic factor Bcl-2 associated X protein (Bax) leading to apoptosis. Therefore, the alteration of the membrane impermeabilization ends up in preventing apoptosis of gastric cells [58]. Inside the mitochondria, VacA can also activate the dynamic related protein 1 which inhibits the activation of Bax mitochondria outer membrane impermeabilization that prevents death by apoptosis[59]. Further, it can also disrupt the gastric epithelial cell-to-cell unions, which prevents T lymphocyte activation and proliferation in the lamina propria, and ultimately induces inflammation and carcinogenesis by disrupting autophagy in gastric cells[60].

In addition, *H. pylori* induces a chronic inflammatory state by upregulating many pro-inflammatory cytokines, like IL-1, IL-6, IL-8, TNF-alfa, and NF-kB[61]. Among them, activation of NF-kB and upregulation of IL-8 are crucial[62]. Suppressor gene P53 also plays an important role in gastric inflammation and carcinogenesis. Mutations of p53 have been related to the development of gastric cancer. In fact, inactivation of p53 gene is present in 40% of gastric tumors and has been specifically found in patients infected with the CagA positive strain of *H. pylori*[63]. *H. pylori* infection has also been related to epigenetic modification during gastric carcinogenesis by promoting hypermethylation of O6-mehylguanine DNA methyltransferase (MGMT). MGMT protein is essential for the repair of O6-methylguanine, which prevents mutations during DNA replication. Therefore, reduced levels of MGMT can increase mutagenesis in the gastric epithelium[64]. Finally, *H. pylori* has been linked to the oxidative stress that promotes DNA damage in gastric tissue by stimulating the production of intracellular reactive oxygen and nitrogen species in gastric tissues, which can damage DNA tumor suppressor genes like p53 and contribute to gastric carcinogenesis[65].

In summary, the *H. pylori* infection is one of the leading factors in the development of gastric carcinoma. Chronic inflammation induced by *H. pylori* is related to cell proliferation, apoptosis, epigenetic changes of tumor suppressor genes, and alterations of the oxidative stress mechanisms. Eradication of *H. pylori*, especially in endemic areas, should be a healthcare priority in order to decrease gastric cancer-related casualties.

GALLBLADER CANCER

Gallbladder cancer (GC) is a common hepatobiliary malignancy that carries very poor prognosis. It is the fifth commonest gastrointestinal tract cancer and is endemic is several countries, with the highest incidence rate reported in Delhi (India), Pakistan and Quito (Ecuador). It shows female preponderance[66], and genetic, infectious, and lifestyle factors have been associated with GC[67]. Infection leads to chronic gallbladder inflammation that seems to contribute to carcinogenesis. Two species-Salmonella Typhi and Helicobacter Pylori- and a liver parasite -Clonorchis sinensis-, among others, have been specifically linked to GC.

Salmonella Typhi

The association between chronic typhoid infection and GC was first reported in 1971 [68] and confirmed by ulterior studies. For example, in 1964, during the typhoid outbreak in Aberdeen (Scotland), Caygill *et al* studied the mortality rate of infected population and reported a 6% of risk of death attributable to GC among chronic carriers[69].

Further studies from Northern India reported a 7.9-fold increased risk of GC among infected persons. Species of Salmonella typhi and paratyphi-A could be isolated from bile, gallbladder tissue and stones in patients with GC. Bacterial serologic detection was related to a 9.2-fold increased risk of developing GC.

Another study from India, using nested PCR technique in hepatobiliary specimens, detected a 67.3% of typhoid carriers among GC patients compared to 8.3% of controls. The authors concluded that the liver served as a preferential survival niche for *Salmonella* Typhi (*S*. Typhi)[70]. As *Salmonella* spp. are excreted in the gallbladder, the multiplying bacteria secrete toxins and metabolites that favor mutational changes. Several carcinogens have been proposed: bacterial glucuronidase, bacterial enzymes and the production of nitrous compounds from nitrates[71,72].

Additionally, *S*. Typhi produces a genotoxin, the cytolethal distending toxin (CDT) that allows bacteria to internalize inside the host cells. Then, they are able to avoid the usual endocytic pathway that leads to the formation of destructive lysosomes and finally reaches a specific intracellular compartment where it can survive and replicate [73]. Once in the cytosol, the CDT facilitates the persistence of infection thanks to its immunomodulatory activity. Later on, it can reach the nucleus causing irreversible DNA damage[74].

Helicobacter spp.

H. bilis, *H. pullorum*, *H. hepaticus,* and *H. pylori* have been suspected to cause biliary tract diseases. *Helicobacter* spp. cause persistent infection in the biliary tract inducing chronic inflammation and gallstone formation, mainly *via* urease production[75,76]. *Helicobacter* spp. can produce several carcinogenic toxins and metabolites that initiate malignant cell transformation within the gallbladder[77]. Helicobacter bilis specific DNA sequences have been amplified in 27.2% of gallbladder and in 33.3% of biliary duct cancers[77].

Escherichia coli

Escherichia coli (*E. coli*) is part of the regular human intestinal microbiota but may become highly pathogenic following the acquisition of virulence factors, like the cytotoxic necrotizing factor I -CDT I-. The presence of genotoxin colibactin, related to *E. coli* infection, is known to be capable of inducing double stranded DNA breaks[10].

Opisthorchis viverrini and Clonorchis sinensis

Liver fluke *Clonorchis sinensis* (*C. sinensis*) is a high-risk pathogenic parasitic helminth endemic in some Asian countries. This parasite is classified among the Group I of human carcinogens by the IARCC[1]. Fresh water snails and several species of fish serve as secondary intermediate hosts. Humans and other fish-eating animals are infected through the ingestion of raw or undercooked freshwater fish that contains

meta-cercariae. After ingestion, the meta-cercaria excysts in the duodenum and ascends the biliary tract through the ampulla of Vater[78].

Infection with *Opisthorchis viverrini* has been related to cholangiocarcinoma upon the results of several cross-sectional and case-control studies[79]. The association between *C. sinensis* infection and cholangiocarcinoma is even better substantiated and the IARCC has already classified it as a probable carcinogenic for humans (Group 2A) [80,81].

Liver fluke pathogens cause mechanical injury and inflammation in the biliary tree, leading to metaplasia of mucin-producing cells, periductal fibrosis and hyperplasia of epithelial cells[82]. The severity of these changes correlates with the duration of the infection, the parasite load and the susceptibility of the host. The molecular mechanisms involved in the development of cholangiocarcinoma are poorly known, and a multistep process has been proposed: chronic inflammation damages cholangiocytes which in turn promotes cell proliferation and genetic and epigenetic mutations, finally transforming cholangiocytes into malignant cells[83]. Other carcinogenetic mechanisms include genomic instability, transcriptomic, proteomic and microRNA profile alterations and dysregulation of immune response.

LIVER CANCER

Worldwide, liver cancer is the sixth most common cancer, yet the second leading cause of cancer-related death in men, with 745000 deaths per year[84]. Hepatocellular carcinoma (HCC) represents approximately 90% of all primary liver cancers. It preferentially affects males and presents a wide geographical variation. However, chronic Hepatitis B virus and HCV infections are the leading cause of HCC, being responsible for 60%-80% of all these tumors worldwide, especially in developing countries.

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that replicates *via* reverse transcription. It is highly contagious and is transmitted by percutaneous and permucosal exposure to infected blood and other body fluids. HBV particles package the incomplete double-stranded DNA into the nucleus of the host, where the virus is recognized as damaged and induces a DNA repair response, resulting in virus replication[85]. Chronic HBV infection is one of the leading causes of HCC worldwide. HBV typically replicates inside the hepatocytes. Virions bind to the surface of the hepatocytes and the nucleocapsid is released into the cytoplasm and translocated by microtubules to the microtubule-organizing center near the nucleus. Once inside the nucleus, HBV can lead to histone degradation, which enhances chromatin dynamics and may promote genetic instability, chromosomal alterations, and can also initiate oncogene mutation[86]. However, HBV integration into the hepatocytes occurs randomly -at one or multiple sites- and occasionally may promote direct oncogene activation or inactivate tumor suppressor genes[87]. While viral integration is an early event, selective clonal amplification of hepatocytes occurs throughout progression of the disease[88]. It is known that both the HBV-encoded envelope and the regulatory HBx protein directly contribute to hepatocyte transformation: HBx regulates expression of many genes involved in signal transduction pathways, cell cycle control, metastases, and avoidance of immune response. Several cohorts and case-control studies have assessed the risk of HCC among individuals infected with HBV[89,90]. The ability of antivirals to inhibit HCC progression is limited, likely because hepatocarcinogenesis seems to occur prior to the onset of liver fibrosis/cirrhosis. Therefore, once the disease is established, there is no turning back.

HCV is a single-stranded, positive sense RNA virus that encodes a single polyprotein that can form structural -which constitute the viral particle, such as the core protein- and non-structural proteins which support viral genomic replication. Seven genotypes have been described. Epidemiological studies show than infection with genotypes 1b and 3 is associated with an increased risk of developing HCC[91]. HCV replicates in the cytoplasm of hepatocytes and is unique among cancer-causing viruses by not encoding oncoproteins or integrating its genome into the host chromosomal DNA. In fact, HCV core genes variants have been associated with HCC even in patients in which the infection had already disappeared[92]. This suggests that viral factors influence progressive liver disease. HCV associated carcinogenesis includes increased hepatocyte proliferation and steatosis; virus-induced inflammation and oxidative stress which induces genomic mutations and genome instability; mitochondrial damage and induction of reactive oxygen species; and inhibition of host immune responses[93]. HCC is associated with the development of multifocal, genetically distinct tumors throughout the liver, suggesting that the entire organ is

altered.

COLON CANCER

Colorectal cancer is the leading cause of mortality regarding gastrointestinal neoplasms worldwide[94]. Pathogenic microorganisms able to induce intestinal dysbiosis have become the spotlight of current research in this area, as they carry the potential for colorectal tumorigenesis. Fusobacterium nucleatum, *E. coli*, *Bacterioides fragilis* (*B. fragilis*) and *Salmonella enterica* (*S. enterica*) have been reported as high-risk oncogenic pathogens.

Fusobacterium nucleatum

Fusobacterium nucleatum (*F. nucleatum*) is an adherent and invasive Gram-negative anaerobic bacterium frequently found in the oral cavity. Current research relates *F. nucleatum* to the development and progression of colon cancer, and has been found in primary lesions and stools of patients with colon cancer[95], especially those located in the cecum and rectum[96]. Tumoral cells over-express Gal-GalNAc molecules, which promote bacterial adhesion through the Fap2 protein[97].

Additionally, *F. nucleatum* infection has been related to a decreased survival rate among colon cancer patients and an increased resistance to chemotherapy agents[98, 99]. The presence of *F. nucleatum* in colon cancer patients varies worldwide, ranging from 15% in North American to 60% in Chinese patients[100]. Interestingly, tumors infected with *F. nucleatum* show three similarities: they are microsatellite unstable, show a methylation phenotype of CpG island, and exhibit mutations in the BRAF/KRAS genes[101]. Microsatellite instability is responsible for the ability of infected cells to elude the immune response[102] and has also been related to the activation of beta-catenin signaling pathway, commonly unregulated in colon cancer [103]. *F. nucleatum* also promotes inflammation, by increasing TNF-alfa and IL-10 Levels in adenomas and Il-6 and IL-8 in carcinomas, both regulated by the NF-kB transcription factor[104].

E. coli

E. coli is a widely distributed Gram-negative bacterium that can alter the intestinal microbiome. The B2 *E. coli* strains have been related to colon cancer[105]. *E. coli* promotes colon pathologic inflammation -as in Chron's disease- which seems to be a relevant factor in colon cancer formation[106]. However, the exact role of *E. coli* in the pathogenesis of colon cancer is not completely known. Recent studies have identified two potentially pathogenic *E. coli* strains: Adherent-invasive *E. coli* (AIEC) and entero-pathogenic *E. coli* (EPEC). During infection, AIEC binds to CEACAM6 (a cellular adhesion receptor associated to carcinoembryonic antigen- CEA- which is overex-pressed in colon cancer cells[107]. Infection stimulates IL6 production, which together with the increased expression of CEACAM6 can promote carcinogenesis. AIEC also secretes colibactin, a secondary metabolite associated to DNA damage acting as an alkylating agent and promoting tumor development[108]. EPEC is thought to stimulate macrophage-inhibitory cytokine-1 production - a cytokine related to metastasis- and inducing autophosphorylation of EGFR receptor[109,110].

B. fragilis

The *Bacteroides* spp. are regularly found in the human intestine and comprises 30% of the microbiota[111]. *B. fragilis* is an anaerobic Gram-negative bacterium colonizing about 0.5%-2% of the entire human intestine. A toxigenic *B. fragilis* strain -also known as ETBF- has a pathogenicity island encoding a metalloproteinase, the *B. fragilis* toxin (BFT) which is associated to increased inflammatory bowel disease and colitis, both considered high risk factors to develop colon cancer[112]. *B. fragilis* has also been detected in stool from cancer patients. Although the exact role of enterotoxigenic *B. fragilis* has not been completely described, it is believed that carcinogenesis is either induced by BFT toxin secretion or through host immune system dysregulation[113]. It can also trigger carcinogenesis through the beta catenin pathway activation by disrupting the adherent e-cadherin gap unions[114].

S. enterica

S. enterica includes serotypes of Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis and Salmonella typhimurium. In recent years, it has been found that bacteria may modulate host immune response in two different ways: First, it can

promote carcinogenesis inducing both DNA damage and increasing cell abnormal proliferation; and second, it can induce cell migration as a result of chronic inflammation[17].

Two proteins of *S. enterica* have been associated with an increased risk of developing colon cancer: typhoid toxin and AvrA effector protein. Typhoid toxin is a cyclomodulin that, similar to *E. coli* CDT, increases cell survival and promotes intestinal dysbiosis, resulting in the development of inflammatory bowel disease and colon cancer[115,116]. AvrA is secreted by bacteria and has been found in stool samples from colon cancer[117]. AvrA may promote inflammatory and immune dysregulation through several mechanisms: the inhibition of NF kappa-beta signaling pathway, the inhibition of IL-12, INF-c and TNF-α secretion, the inhibition of IL-6 transcription, and the increase of IL-10 transcription[118,119]. On the other hand, AvrA might also activate the Wnt/β catenin pathway inducing cellular proliferation, by both β catenin phosphorylation and deubiquitination[120].

ANAL CANAL

Anal cancer refers to the malignancy of the intestinal mucosa arising in the anatomic anal canal, defined as beginning at the dentate line and ending at the anal verge. Eighty-five percent of anal tumors are of squamous cell origin, approximately 10% are adenocarcinomas, and the remaining 5% are other neoplasms (melanoma, small cell carcinoma…)[121]. Although it comprises 2.7% of all gastrointestinal malignancies, anal cancer incidence has been increasing over the last few decades[122].

As previously highlighted in the paper, HPV are small DNA viruses that infect various epithelial tissues. They also include the anogenital tract, and HPV is recognized as the causative agent of more than 80% of cases of anal cancer[123]. The difference in their ability to promote malignant transformation is the basis for the classification of HPV into low and high-risk variants. Mainly two HPV oncogenic subtypes, 16 and 18, are related to the development of squamous anal cancer[124]. HPV can integrate into the host DNA. Epithelial cells that harbor integrated HPV 16 DNA have a selective growth advantage over cells that carry normal extrachromosomal viral genomes; this growth advantage correlates with the increased expression of two viral genes in particular, E6 and E7[125]. The early proteins, E6 and E7, bind and inactivate the tumor-suppressor gene p53, and the retinoblastoma tumor-suppressor protein (pRb), respectively[126-128].

GUT MICROBIOTA

Most of the literature summarized in the present paper has been published previous to 2016 as research over the last years is focused in the role of gut microbiota in human carcinogenesis. Although it is not the scope of our review, we cannot conclude our review without summarizing intestinal microbiota key points. Gut microbiota consists of viruses, fungi and more than 1000 bacteria which are crucial for maintaining the gut barrier, metabolism and immunity. Disruption of the host relationship with the microbiota might result in oxidative stress, chronic inflammation and dysbiosis which can finally promote carcinogenesis[129]. The influence of gut microbiota relies mainly on cancers in the gastrointestinal tract, and the regulation of microbiota by diet, prebiotics, probiotics, symbiotics and antibiotics are proposed as new strategies to be explored in the future[130]. Gut microbiota is thought to play an important role in colon -stool samples of patients with CRC have higher proportions of Escherichia and Fusobacterium and lower concentrations of Firmicutes and Actinobacteria[131], HCC [132] and GC[133], Interactions between gut microbiota and GI cancers are likely to yield new opportunities to reduce cancer morbidity and mortality (Table 1).

CONCLUSION

Cancers arise from the transformation of a single cell so that its behavior is no longer under the control of normal regulatory pathways. The relationship between some pathogens and gastrointestinal tumor carcinogenesis has been well stablished. However, this relation is difficult to determine with many other agents as cancer is a multistep process. Besides, the presence of bacteria at the site of a tumor does not itself

implies causation. There is a long time period between the onset of carcinogenesis and the development of overt disease, and randomized studies are expensive and extremely difficult to make (one cannot, for example, infect a person with an agent and then wait to see if cancer develops). However, the occurrence of cancer attributable to infections is a global concern especially in underdeveloped regions. Chronic infections can mimic precancerous lesions that could be treated with antibiotics or antiviral treatment and therefore prevent the onset of carcinogenesis. Greater understanding of the consequences of long-term infections will help to elucidate the exact pathogenic processes involved in the development of some pathogen-related neoplasms. Preventive measures (like vaccines and antimicrobial agents) aimed to eradicate these oncogenic microorganisms should ideally interrupt the carcinogenetic pathways and avoid the formation of a relevant proportion of gastrointestinal cancers. Only global effort and an international strategy might help to decrease gastrointestinal cancer related deaths secondary to infectious pathogens. New molecular techniques are needed to identify new infectious agents in tissues. However, caution is requested not to overemphasize the association between pathogens and gastrointestinal malignancies without a proper causation proof.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 522-542

DOI: 10.13105/wjma.v9.i6.522

ISSN 2308-3840 (online)

REVIEW

Newer developments in viral hepatitis: Looking beyond hepatotropic viruses

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Author contributions: Gupta M, Manek G and Dombrowski K were involved in the study conceptualization, data curation, funding acquisition, investigation, project administration, and writing of the original draft; Maiwall R performed the review and editing of the manuscript.

Conflict-of-interest statement: No conflict-of-interest for any of the authors.

Country/Territory of origin: United States

Specialty type: Gastroenterology and Hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Open-Access: This article is an

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Abstract

Viral hepatitis in the entirety of its clinical spectrum is vast and most discussion are often restricted to hepatotropic viral infections, including hepatitis virus (A to E). With the advent of more advanced diagnostic techniques, it has now become possible to diagnose patients with non-hepatotropic viral infection in patients with hepatitis. Majority of these viruses belong to the Herpes family, with characteristic feature of latency. With the increase in the rate of liver transplantation globally, especially for the indication of acute hepatitis, it becomes even more relevant to identify non hepatotropic viral infection as the primary hepatic insult. Immunosuppression post-transplant is an established cause of reactivation of a number of viral infections that could then indirectly cause hepatic injury. Antiviral agents may be utilized for treatment of most of these infections, although data supporting their role is derived primarily from case reports. There are no current guidelines to manage patients suspected to have viral hepatitis secondary to non-hepatotropic viral infection, a gap that needs to be addressed. In this review article, the authors analyze the common non hepatotropic viral infections contributing to viral hepatitis, with emphasis on recent advances on diagnosis, management and role of liver transplantation.

Key Words: Hepatitis; Non hepatotropic viruses; Cytomegalovirus; Herpes simplex virus; Coronavirus-2019; Liver transplant

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Received: July 16, 2021
Peer-review started: July 16, 2021
First decision: August 9, 2021
Revised: September 9, 2021
Accepted: December 8, 2021
Article in press: December 8, 2021
Published online: December 28, 2021

P-Reviewer: Cao HC
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M



Core Tip: With growing numbers of patients receiving solid organ transplantation including liver transplant, and subsequent immunosuppression there is an increasing incidence of non-hepatotropic viruses causing hepatitis. Several gaps exist in the diagnosis and management of such patients. Through this review article we attempt to outline the important non hepatotropic viruses causing liver injury. We also address the challenges in diagnosis, current and future prospects in treatment as well as prevention of these infections.

Citation: Gupta M, Manek G, Dombrowski K, Maiwall R. Newer developments in viral hepatitis: Looking beyond hepatotropic viruses. *World J Meta-Anal* 2021; 9(6): 522-542
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/522.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.522

INTRODUCTION

Viral hepatitis in the entirety of its clinical spectrum is vast and most discussion are often restricted to hepatotropic viral infections, including hepatitis virus (A to E). With the advent of more advanced diagnostic techniques, it has now become possible to diagnose patients with non-hepatotropic viral infection in patients with hepatitis. Non hepatotropic viruses do not infect the liver as the primary organ. These infections can present as hepatitis as a part of systemic infection. In a study performed in India, 10.5% of the patients with acute hepatitis and acute on chronic hepatitis were found to be secondary to non-hepatotropic viral infection[1]. Majority of these viruses belong to the Herpes family, with characteristic feature of latency. With the increase in the rate of liver transplantation globally, especially for the indication of acute hepatitis, it becomes even more relevant to identify non hepatotropic viral infection as the primary hepatic insult. Immunosuppression post-transplant is an established cause of reactivation of a number of viral infections that could then indirectly cause hepatic injury. Antiviral agents may be utilized for treatment of most of these infections, although data supporting their role is derived primarily from case reports. There are no current guidelines to manage patients suspected to have viral hepatitis secondary to non-hepatotropic viral infection, a gap that needs to be addressed. In this review article, the authors analyze the common non hepatotropic viral infections contributing to viral hepatitis, with emphasis on recent advances on diagnosis, management and role of liver transplantation.

ETIOLOGY OF VIRAL HEPATITIS

The most important and common cause of viral hepatitis is infection with hepatotropic virus, including hepatitis A-E. However, a small percentage of individuals exhibit signs and symptoms of hepatitis without testing positive for any of the hepatotropic viruses. In such patients, the differential diagnosis should be expanded to include other non-hepatotropic virus, listed in Table 1.

CYTOMEGALOVIRUS

Epidemiology

Cytomegalovirus (CMV) or human herpes virus-5 (HHV-5) belongs to the herpesvirus family. It is an enveloped, double-stranded DNA virus that remains latent in the body in two-thirds of the patients after primary infection. The capacity of the virus to remain latent in the host cells leads to risk of endogenous reactivation in a susceptible host, in addition to the risk of exogenous transmission. It is one of the most common viruses causing chronic infections as reflected in seroprevalence rates (ranging from 40%-100%) in adults and increases with age[2]. Demographic variability exists; women, non-white population and people belonging to the lower socioeconomic strata exhibit a higher prevalence[3-5]. CMV infection and disease are defined as distinct entities. CMV infection is any evidence of replication of the virus regardless of

Table 1 Example of non-hepatotropic viral infection causing hepatitis

Herpesvirus	HSV1, HSV2, HHV6, HHV7, HHV8, EBV, CMV, VZV
Adenovirus	
Enterovirus	Coxsackie B virus, Echovirus
Paramyxovirus	Measles
Togavirus	Rubella
Parvovirus	Parvovirus B19
Coronavirus	COVID-19

COVID-19: Coronavirus disease 2019; HSV: Herpes simplex virus; HHV: Human herpes virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus.

symptoms whereas CMV disease is infection along with symptoms that are explained by the virus[6]. CMV hepatitis is exceedingly rare in immunocompetent individuals and is more prevalent in immunocompromised patients, particularly post liver transplant (LT)[7]. Based on data available from population-based studies, 1%-4% of adults with acute hepatitis are due to CMV in developed countries[1].

Pathogenesis and clinical features

The transmission of CMV is through multiple routes including sexual exposure and close contact with bodily fluids such as saliva and breast milk[8]. The virus initially infects mucosal epithelial cells, and has broad cellular tropism allowing it to interact with a myriad of cell surfaces. Systemic dissemination occurs hematogenously with polymorphonuclear leukocytes, macrophages in the gastrointestinal and pulmonary tissues, and infected monocytes, all playing a role[9-12]. CMV has a predilection for hematopoietic, connective tissue and parenchymal cells; it specifically infects hepatocytes and macrophages in the hepatic tissue[11,13]. The virus then has complex interactions with the immune system leading to the repression of the primary infection, which is often followed by the stage of latent infection[14]. It plays a role in modulating both the humoral and adaptive immune responses in humans[15]. The sinusoidal endothelial cells of the liver, instead of being a barrier, provide an ideal environment for viral dispersion through the organ and act as sites for latency and reactivation[16,17]. The sinusoidal cells also facilitate immune activation in the liver by modulating T cell recruitment and activation *via* trans endothelial migration of CXCL10 and ICAM-1 dependent CD4+ T cells[18]. Notably, the sinusoidal cells play a role in viral latency, reactivation and dissemination within the liver but have a limited capacity for viral replication. Hepatocytes, on the other hand, play a major role in viral reproduction but have a limited role in latency. The pathogenesis of CMV disease is summarized in Figure 1.

Various factors lead to the reactivation of the virus such as allogeneic transplantation (especially those receiving anti-lymphocytic drugs), ischemia/ reperfusion, sepsis, immune cell depletion, injury and other inflammatory states[19].Immunosuppressant medications like corticosteroids and cyclosporine do not directly cause reactivation but can facilitate viral replication[20-22]. Allograft rejection is an important risk factor as well a consequence of CMV disease[22,23].

CMV causes indirect cytotoxicity in the liver *via* cytotoxic T cell activation and alterations in vasculature, subsequently causing necrosis[24-26]. Additionally, it also has a direct cytotoxic effect on hepatocytes as evidenced in a study by Sinzger *et al*[11] that demonstrated lysis of CMV infected hepatocytes. Thus, in contrast to other herpetic infections such as Epstein-Barr virus (EBV), CMV affects the liver both indirectly *via* continuous immune activation and cytokine release as well as with direct cytotoxicity.

The clinical features vary according to the patient's immune status. Both stages, acute and chronic stages, are seen with the viral infection. In immunocompetent patients, a mononucleosis-like syndrome is seen with splenomegaly and hepatic dysfunction. Only case reports exist describing uncommonly seen CMV hepatitis in immunocompetent hosts[11,13]. Immunocompromised patients, especially LT patients, have a high incidence of CMV tissue invasive disease including hepatitis, esophagitis, gastritis, enteritis and/or colitis[21,23,27]. The risk of CMV hepatitis occurs with the highest frequency in the combination of seropositive donor/ seronegative recipient patients (incidence estimate of 44%-65%), followed by the

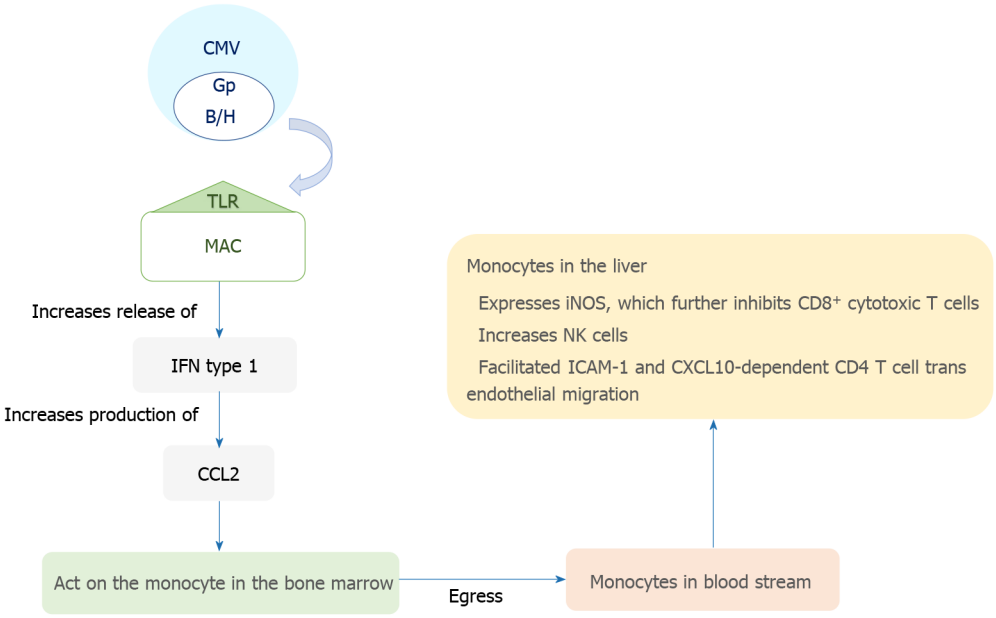


Figure 1 Pathogenesis of cytomegalovirus disease in the liver. CMV: Cytomegalovirus; Gp: Glycoprotein; TLR: Toll-like receptors; MAC: Macrophages; IFN: Interferon; CCL2: Cytokine; Inos: Inducible nitric oxide synthase.

combination of seropositive donor/seropositive recipient patients or seronegative donor/seropositive recipient (8%-18%), and with the least frequency in the combination of seronegative donor/seronegative recipient patients (1%-2%)[23,28].

A study by Toghill *et al*[29] studied 70 patients with cirrhosis due to a variety of causes including alcoholic cirrhosis, primary biliary cirrhosis, secondary biliary cirrhosis, hemochromatosis, congenital hepatic fibrosis and cryptogenic cirrhosis. The authors did not find evidence of CMV disease as the cause for the liver cirrhosis and the antibody titers in these patients were similar to that of the general population. CMV disease was, however, found to be an important cause of chronic rejection in post-LT patients and associated with increased mortality in patients with cirrhosis[30-32]. Liver involvement in CMV is varied and can manifest as mild hepatitis, necrotizing hepatitis, granulomatous hepatitis or even portal vein thrombosis.

Diagnosis and treatment

The diagnosis of CMV is starts with serological testing to detect CMV IgM and IgG antibodies, antigenic testing of CMV pp65 that detects CMV antigens in leukocytes, polymerase chain reaction (PCR), culture and biopsy. Serological testing can provide risk assessment prior to LT but its role is limited in the diagnosis of CMV in immunocompromised patients due to the inability of these patients to mount an immune response[20,33,34]. In immunocompetent patients, serological tests may be falsely positive due to cross reactivity with other herpetic viruses, persistence of antibody levels after primary infection, reactivation or presence of rheumatoid factor[32,35].

Serological tests may provide quick diagnosis in immunocompetent patients after other etiologies of hepatitis have been ruled out. The pp65 antigen assay has a sensitivity of 64% and a specificity of 81% but since it detects antigens in leukocytes, it may not be reliable in patients with leukopenia[35,36]. The utility of viral culture is limited due to the long turnaround time, with one study demonstrating sensitivity of only 52% with cell culture[37]. The use of shell vial assay has the advantage of faster turnaround time (12 h), similar specificity to traditional culture and higher sensitivity [38]. PCR has a high sensitivity and specificity, ranging from 61%-92% and 75%-99%, respectively[39,40]. It can provide both quantitative and qualitative measurements from body fluids or tissue samples and is particularly useful in immunocompromised patients to determine the need for preemptive therapy and monitoring disease response[35,41]. While liver biopsy is not mandatory for diagnosis, it may be required when the diagnosis is uncertain. It is also required in LT patients to distinguish between acute graft rejection and CMV infection since CMV is a risk factor for rejection[8,28]. CMV hepatitis has characteristic histology with cytoplasmic and intranuclear inclusion bodies, nonspecific hepatocellular necrosis, mononuclear cell infiltrate and micro abscesses[42,43]. The degree of inflammation on the biopsy

depends on the immune status of the patient. To increase the sensitivity, immunohistochemistry and/or DNA hybridization can be added to the liver biopsy[6,44].

Agents acting on CMV DNA polymerase including ganciclovir, valganciclovir, foscarnet and cidofovir are recommended for treatment of CMV hepatitis in immunocompromised individuals[6,28]. Immunocompetent patients usually have self-limited infectious mononucleosis (IM) like syndrome which does not require treatment. In immunocompetent patients with severe disease, limited data suggests using the above mentioned anti-viral agents[45-47]. There also have been reports of acute liver failure (ALF) from CMV hepatitis requiring LT[47-49].

LT

About 18%-29% patients receiving LT are affected by CMV disease and it remains one of the most common infectious complications following solid organ transplant (SOT)[28,50]. Infection in the LT recipient can either be a primary infection, re-infection or reactivation of the latent virus. CMV disease in LT patients leads to other co-morbidities such as acute or chronic rejection, graft loss, post-transplant lymphoproliferative disorders (PTLD), increased infections, vascular thrombosis and increased mortality[28,51]. Increased rates of bacterial infection, invasive fungal infection such as Nocardia and viral co-infection such as EBV, HHV6, HHV7 and HCV has been described in literature[28,52-54].

Two basic approaches have been proposed to prevent CMV disease post-liver transplantation: Prophylactic and pre-emptive. The prophylactic approach refers to treatment which is immediately started post-transplant and continued for three to six months while the pre-emptive therapy refers to close monitoring for evidence of CMV replication with prompt initiation of antiviral therapy upon detection[23]. Both approaches have been shown to have comparable efficacy [0.34, 95% confidence interval (CI): 0.24-0.48 with prophylactic approach *vs* 0.30, 95%CI: 0.15-0.60 with preventative approach] in a meta-analysis. Notably, the population used in this meta-analysis was treated with ganciclovir as opposed to preferred alternative, valganciclovir[55].

For high-risk recipients (seropositive donor/seronegative recipient), prophylactic therapy is preferred with acyclovir, valacyclovir, intravenous ganciclovir and valganciclovir, if available for use[6,22]. Valganciclovir has demonstrated better efficacy, lower incidence at 6 mo and 12 mo follow up and better safety profile in multiple studies[21-23]. Preemptive therapy requires resource intensive monitoring which may not be achievable in all clinical settings. It can still be employed for high-risk LT patients (seropositive donor/seronegative recipient) and intermediate risk LT patients (seropositive donor/seropositive recipient, seronegative donor/seropositive recipient). Intermediate risk LT patients can also be managed with prophylactic therapy[13]. Low-risk LT patients (seronegative donor/seronegative recipient) do not require routine prophylaxis. Table 2 outlines the strategies for CMV prevention in LT patients based on risk stratification.

Ongoing research and future directions

Another high-risk patient population for CMV disease are patients undergoing hematopoietic stem cell transplant patients (HSCT). This field is rapidly evolving with ongoing research on multiple strategies for management of disease and risk mitigation. The concept of adoptive transfer of T-cells with protective effects against CMV is currently being studied[56-58]. Letromovir, a viral terminase complex inhibitor, has been approved for prophylactic CMV treatment for HSCT transplant patients and acts against both viral replication as well as latent infection[59]. Maribavir, an inhibitor of the viral kinase UL97, is also being evaluated in patients undergoing HSCT and has shown better safety profile with regards to hematologic side effects as well as nephrotoxic effects when compared to ganciclovir and valganciclovir[60]. A phase III trial comparing maribavir and placebo did not show any difference in patients with HSCT[61]. However, the trial used low-dose maribavir and repeating the trial with higher doses may reveal different, perhaps, positive results[62]. Maribavir is also being evaluated in an ongoing phase III clinical trial as a treatment for CMV disease in transplant recipients with resistance to ganciclovir, cidofovir and foscarnet (NCT02931539). The therapies used in HSCT patients may have a future in patients undergoing liver transplantation, given the overlap in immune status. Therapies against CMV latency can have significant clinical benefits. As indicated by *in vitro* studies, vincristine has the potential to be a therapeutic agent with the ability to kill latent infected cells; however, its use is limited by the extensive adverse effect profile[63]. A protein named F49A-fusion toxin protein (FTP) which kills infected cells has been developed, which may be a possible future therapeutic agent to

Table 2 Strategies for cytomegalovirus prevention in liver transplant patients based on risk status

Risk status	Donor/Recipient CMV serological status	Prevention strategy
High risk	Donor positive/recipient negative	Prophylactic therapy for 3-6 mo
		Or
		Pre-emptive therapy requiring close monitoring
Intermediate risk	Donor positive/recipient positive	Prophylactic therapy for 3 mo
		Or
		Pre-emptive therapy requiring close monitoring
Intermediate risk	Donor negative/recipient positive	Prophylactic therapy for 3 mo
		Or
		Pre-emptive therapy requiring close monitoring
Low risk	Donor negative/recipient negative	No routing prophylaxis

CMV: Cytomegalovirus.

target latent disease[13,64]. Apart from this, studies have also suggested using immunotherapeutic strategies which force the virus to be partially reactive only to be detected and demolished by the host immune system[14,65].

Several vaccine candidates have been developed including live attenuated viral vaccines, and subunit vaccines against CMV phosphoprotein 65 and glycoprotein[13,66]. Till date, the most efficacious results are from a subunit recombinant vaccine against CMV glycoprotein with MF59 adjuvant indicating 50% efficacy in young mothers as well as in recipient negative/donor positive transplant patients[67].

EBV

Epidemiology

The most common presentation of primary EBV is IM which manifests as fever, cervical lymphadenopathy, tonsillitis and splenomegaly. In 90% of these cases, abnormal liver function tests are noted with hepatomegaly observed in about 14% cases[68]. However, a much smaller percentage of the population, estimated to be 0.85%-1% in population-based studies, are diagnosed with EBV hepatitis[69,70]. According to the available literature, the incidence of ALF secondary to EBV is estimated to be 0.21%[71]. In a recently published Russian study, EBV DNA was detected in 58.1% of the patients with viral hepatitis and correlation indicated worse outcomes in hepatitis C patients, coinfected with EBV[72]. The median age for EBV hepatitis in a British population-based study was noted to be 40 years and 41% of the individuals were above the age of 60 years[70]. Subsequently, another population-based study indicated the median age of patients to be 17 years, overlapping the age group most commonly affected by IM[69]. The scarcity of data and the difficulty in determining causation of EBV in patients with viral hepatitis or hepatitis of unknown etiology stems largely from lack of a diagnostic criteria. This forms the basis for the need to develop better diagnostic tools to identify these patients and initiate early treatment.

Pathogenesis and clinical features

EBV or herpes human virus 4 belongs to the family of herpesvirus and has predilection for epithelial cells of the oropharynx and B lymphocytes. Once the virus infects B lymphocytes, it causes polyclonal expansion of T lymphocytes (specifically cytotoxic CD8 T cells). As EBV does not directly infect hepatocyte, vascular or biliary epithelium, the primary mechanism of damage is mediated indirectly through cellular immune responses. In majority immunocompetent patients (approximately 90%), hepatic involvement is subacute, mild, anicteric and self-limiting. In rarer cases, despite immunocompetence, the involvement can be acutely severe, recurrent or chronic[69,70]. In immunocompromised individuals, severe hepatitis with icterus is more commonly seen[72].

Another important concern in immunocompromised individuals following transplantation is the development of PTLD. EBV has been recognized as the cause for development of PTLD in 70% cases and occurs due to unregulated replication of EBV infected B cells in an environment of T cell immunosuppression. Depending on the source of EBV infected B cells that generate the clone pathognomic of PTLD in these patients, the disorder can be classified as host-derived PTLD and donor-derived PTLD. In patients receiving hematopoietic stem cell transplant, PTLD is often systemic and secondary to activation of latent EBV infection in the host[73,74]. Following LT, one study showed latent EBV infection in the donor as a likely cause[75]. The clinical manifestations range from constitutional symptoms to extra nodal lymphadenopathy and organ dysfunction (including allograft dysfunction)[76].

Liver involvement as a result of EBV infection can also be a manifestation of hemophagocytic lymphohistiocytosis (HLH)[77-79]. This rare life-threatening clinical entity occurs as a result of excessive immune system activation, primarily of lymphocytes and macrophages, that results in severe cytopenia, coagulopathy and splenomegaly in addition to hepatitis[80].

Diagnosis

Liver enzymes, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) are elevated up to 5-fold in majority of patients with subacute hepatitis presentation[81]. In the rare case that acute severe hepatitis develops, transaminase levels can exceed 5 times the upper normal limit. Serum bilirubin levels are elevated in only 5%-10% of the patients[68]. Cholestatic pattern of injury [elevated alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT)], compared to other viral etiologies, is seen in some patients with EBV[70]. As part of initial blood work, lymphocytosis with atypical lymphocytes is characteristically seen[82]. In the subgroup with HLH, additional laboratory abnormalities of note are bicytopenia (92% patients), hyperferritinemia (> 500 mcg/L in 94% patients) and hypofibrinogenemia (90% patients)[83]. Heterophile antibodies, although nonspecific, is rapid and has reasonable sensitivity ranging from 85%-100%, depending on assay used. The Paul Bunnell test (against sheep erythrocytes), the Monospot test (against horse erythrocytes) and the enzyme linked immunosorbent assay against other substrates such as ox or goat erythrocytes are some examples of widely available confirmatory tests for EBV infection[84]. In individuals with negative heterophile test but high clinical suspicion, further testing with specific antibody assays against EBV can be used. The immunogenic components of EBV used as basis for antibody testing are viral capsid antigen (VCA) and EBV nuclear antigen (EBNA). Given that 90%-95% of the general adult population in the United States is seropositive for anti-VCA IgG, it is difficult to use it as a diagnostic test in clinical practice[81]. The presence of anti-VCA IgM antibodies in the serum is considered to be a more reliable marker of active EBV infection and lasts for 4-6 wk after infection. IgG antibodies against EBNA, on the other hand, are established 6-12 wk after infection and are a marker for latency or convalescence. Thus, the combination of presence of anti-VCA IgM antibodies and with the absence of anti-EBNA-1 IgG antibodies is key to diagnosis of active EBV infection[85].Additionally, autoantibodies such as anti-nuclear antibodies, anti-smooth muscle antibodies may be seen in EBV infection due to cross reactivity of EBV proteins with cellular antigens. As a result, in immunocompromised individuals, autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus could hypothetically be triggered, further confounding the etiology of hepatitis[86].

In patients diagnosed with IM, the presence of elevated transaminase is sufficient to diagnose EBV hepatitis. However, the diagnosis of isolated EBV hepatitis in the absence of IM is trickier. Liver biopsy is indicated in these patients to establish etiology. The interpretation of the biopsy is challenging as a small percentage of EBV infected lymphocytes may be present in the liver in seropositive individuals without hepatitis. The diagnosis, thus, requires serum testing to establish the context for interpretation of the histopathological features in liver biopsy. Typically, portal and intra-sinusoidal lymphocytic infiltration (B and T cells) with few apoptotic cells is seen in EBV hepatitis. The most common lymphocytic population visualized in EBV hepatitis was CD3 positive cytotoxic T cells[71,87]. The diagnosis is further confirmed by either EBV-DNA PCR or EBER-RISH (EBV encoded RNA in situ hybridization), both methods demonstrating comparable sensitivity[88].

Management

In an analysis published in 2015, ALF secondary to EBV was shown to have a high case fatality rate. While the study population was treated with antivirals (acyclovir, famciclovir and ganciclovir) and high dose steroids, the efficacy of either treatment

option (alone or in combination) is not clearly established[89]. Antivirals such as ganciclovir have shown efficacy in both immunocompromised and immunocompetent individuals[90]. Oral valganciclovir has also been used in immunocompetent individuals, although with uncertain benefit[91]. While steroids have been used in acute hepatitis to limit inflammatory response, steroid use has been proposed to be associated with EBV reactivation, most likely at the time of withdrawal of high dose steroids. This mechanism is possibly the rebound increase of suppressed cytotoxic T cells that attack infected B cell infiltrate in latent EBV[92].

The definitive treatment for management of ALF currently remains LT. Orthotopic liver transplantation has been described in cases of fulminant hepatitis secondary to EBV infection[89,93]. Subsequent treatments with antivirals such as acyclovir has been suggested in a few case studies of patients developing fulminant hepatitis requiring orthotopic liver transplantation. The rationale behind it is similar to the concern outlined with steroid use in management of ongoing hepatitis. While immunosuppression is required post-transplant, reinfection of graft liver remains a possibility, warranting antiviral therapy[94].

Another important concern related to transplantation, both liver and HSCT, is the risk of development of PTLD. This risk can be lowered by cautious use of immunosuppression post-transplant (in terms of dosage, duration and choice of drug regiment), careful donor-recipient matching (in term of avoiding serodiscordant match) and antiviral prophylaxis. While there is no clear data supporting efficacy of antiviral prophylaxis for EBV in adults, oral acyclovir and intravenous ganciclovir have been used in patients receiving liver allograft. The milder spectrum of PTLD is seen to resolve with the cessation of tacrolimus[95]. In patients who cannot tolerate tapering or changing of immune suppression regiment or persist to have PTLD despite cessation of immune suppression, treatment with anti CD20 agent rituximab has shown success. Single agent treatment with rituximab has shown 40%-50% remission. Other therapeutic options for these patients include surgical removal of affected organ (if localized PTLD) or chemotherapy[96-101].

HERPES SIMPLEX VIRUS

Epidemiology

Herpes simplex virus (HSV) 1 and 2 affect the majority of adults in the western world with prevalence being 80% and 30% respectively. Like other members of the Herpesviridae family, the virus exhibits latency in the human body persisting in the neurons. Majority of patients who suffer from HSV hepatitis are immunocompromised such as organ transplant recipients, patients on immunosuppressive medications, patients with acquired immuno-deficiency syndrome, neonates and pregnant women in their second and third trimesters[42,102,103]. A study on HSV hepatitis with 137 patients revealed 24% patients were immunocompetent, 23% were pregnant and 53% patients were taking immunosuppressant medications either for organ transplantation or for other reasons[42,104]. HSV hepatitis can also affect immunocompetent patients[105]. Interestingly there have been case reports suggesting reactivation of latent HSV by inhaled anesthetic agents such as enflurane, isoflurane, desflurane and nitric oxide [105,106].

Pathogenesis and clinical features

A multitude of theories exist regarding HSV pathogenesis in causing hepatitis. As herpes is known to be a neurovirulent virus, studies have shown hepatovirulent strains of HSV that can cause fulminant hepatitis. Another theory suggests an acute infection superimposed on a latent HSV reactivation as causing liver failure. With regards to viral dissemination to the liver, one hypothesis suggests that the virus spreads to the liver from the herpetic lesions in the setting of impaired immunity and delayed type hypersensitivity reaction. While another suggests that during initial infection, a large inoculum of the virus may overwhelm the innate host defenses leading to dissemination to the visceral organs including the liver[107,108].

HSV hepatitis occurs during the primary infection and rarely as a reinfection in immunocompromised individuals. It presents with non-specific features such as fever, abdominal pain in the right upper quadrant, nausea/vomiting with jaundice rarely present. The characteristic herpetic skin rash is present in only about 18% to 50% of patients. Patients also present with leukopenia, thrombocytopenia, markedly elevated liver enzymes, and mild bilirubin increase[103]. Cases of fulminant hepatitis present with aminotransferase levels 50 to 100 times the upper limit of normal.

Patients may also develop acute kidney injury, disseminated intravascular coagulation, multi organ failure and eventually death. Up to 6% of fulminant hepatitis is associated with HSV with favorable outcomes after treatment[109]. With regards to viral related ALF, up to 2 % of cases are attributed to HSV hepatitis and less than 1% of all ALF are due to HSV[110]. These patients typically have a high mortality of up to 90%[111]. Risk factors associated with increased mortality are age > 40 years, immunocompromised status, coagulopathy, encephalopathy, degree of AST elevation and male gender[104].

Diagnosis and treatment

A thorough physical examination of the skin and pelvis should be conducted in patients with suspicion for HSV infection to detect characteristic herpetic lesions. HSV serology (IgM and IgG antibodies) have limited utility due to false negative and false positive results. PCR of HSV DNA utilizing blood samples is rapid, with a better yield than serology and even viral cultures[112,113]. A liver biopsy is imperative in the diagnosis of HSV hepatitis with typical biopsy findings of intranuclear inclusions (Cowdry Type A) occurring in the foci of coagulative or sometimes extensive hemorrhagic necrosis which are irregular in distribution[103]. There is a characteristic scarcity of inflammatory cells in the portal veins or the parenchyma under light microscopy[107]. Due to risk of increased bleeding with the percutaneous approach in patients with ALF, a trans jugular approach is preferred with consideration of administering factor VII recombinant to reduce the risk[113-115]. Computed tomography may reveal diffuse hypodense lesions along with hepatomegaly due to areas of focal necrosis but this is a nonspecific finding also seen in candida hepatitis, lymphoma, sarcoidosis. However, the clinically acute course along with the characteristic skin rash (if present) can help[116-118].

The disease is curable and carries a high mortality, hence treatment must be initiated as soon as possible. While no standardized guidelines or prospective studies exist, literature exists that has shown reduction in mortality and the need for LT from 88% to 51% in patients receiving treatment[104]. The most important aspect is that in patients with high suspicion of HSV hepatitis, empiric acyclovir should be considered until it is ruled out *via* PCR and/or biopsy. Cidofovir and foscarnet can be used in cases of acyclovir resistance which are quite uncommon about 0.27% in immunocompetent patients and 7% in immunocompromised patients[119,120]. Expert consensus recommends treatment from 2 wk to up to 4 wk[104]. Very limited data exists on the use of therapeutic plasmapheresis which theoretically works by removing infectious particles, reducing viral load and buying time for the immune system to mount a stronger response[121].

LT

An urgent LT is indicated in patients not responding to antiviral therapy as above as a final treatment option. Although disseminated HSV is not a contraindication for transplant, thorough evaluation is necessary since sepsis, and multi organ failure is usually present in these cases which can make it difficult to initiate an immunosuppressive regimen post-transplant. Patients who do receive transplant have a higher risk of HSV recurrence , and require life-long acyclovir which contributes towards acyclovir resistance[119,122,123].

In patients who have received LT, HSV tends to occur in the early post-operative period from 20 ± 12 d and is associated with increased mortality[124]. The early recurrence of HSV in LT patients may be due to acquisition of the virus from the donor or due to immunosuppression. A very high index of suspicion is to be maintained since acute cellular rejection or biliary complications are the commonest issues in the early post-operative period. Early diagnosis improves survival and patients should empirically be started on acyclovir as soon as the suspicion arises[125,126]. In patients with LT who are not receiving CMV prophylaxis which also has activity against HSV, prophylactic treatment is associated with low incidence on clinical disease[127].

Ongoing research and future directions

The first attempt at the HSV vaccine was in 1964 by Kern and Schiff[128]. Since a live attenuated vaccine was developed for varicella zoster virus, a member of the alphaherpesvirus, there was a possibility to develop a vaccine against HSV-2 as well[129]. Currently no effective vaccine exists for HSV-2, however, Heprevac- a truncated glycoprotein D2 (gD2) vaccine did show efficacy for prevention of genital HSV-1 disease (58%) and HSV-1 infection (32%) in a clinical trial[130].

Various types of vaccines including whole killed virus, attenuated virus, subunit vaccines (glycoprotein) as well as DNA based vaccines have been attempted to come up with a preventative/therapeutic vaccine against HSV-2[131]. A promising candidate comprising of HSV-2 glycoprotein D2 and infected cell particle 4 mice with matrix-M2 adjuvant provoked a humoral as well as a cell mediated response with acceptable safety profile in a clinical trial. Antiviral therapy along with the above-mentioned vaccine seems to be a promising approach for HSV-2 treatment[132].

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 OR CORONAVIRUS DISEASE 2019

Epidemiology

The coronavirus disease 2019 (COVID-19) pandemic has ravaged the world affecting over 3 million people worldwide, as of July 12, 2021. Case studies from China, where the pandemic emerged, indicated that 2%-11% patients affected by COVID-19 had prior liver comorbidities and abnormal levels of liver enzymes (ALT and AST) were seen in 14%-53% cases[133]. Prothrombin time abnormalities signifying synthetic function of liver were also seen in COVID-19 patients with gastrointestinal symptoms [134]. Another large study of 1099 patients across 552 hospitals in China demonstrated that patients with severe COVID-19 infection had abnormal liver enzyme levels as compared to those with less severe disease[135]. Li *et al*[136] conducted a study among COVID-19 patients and found that patients with elevated C-reactive protein levels greater than 20 mg/L and lymphopenia with counts less that 1.1×10^9 per liter were related to ALT elevation thus highlighting the fact that COVID-19 disease severity correlates with liver dysfunction[136].

Pathogenesis and clinical features

The pathogenesis of COVID-19 induced liver injury continues to evolve as we learn more about the virus. The virus is known to cause immune dysregulation causing systemic inflammatory response syndrome which causes release of inflammatory mediators including interleukins causing a cytokine storm causing hepatocellular injury with the intrahepatic cytotoxic T cells as well as Kupffer cells playing a role[137, 138]. The virus probably also has direct cytotoxic effect but the ACE2 receptors, which the virus has an affinity for, is expressed in the bile duct cells more than the hepatocytes[139]. It would thus be expected that patients would have elevated ALP levels but patients with COVID-19 hepatitis usually have elevated AST and ALT levels. The virus predominantly affects the lung and in severe disease causes refractory hypoxemia as well as hypotension leading to ischemic liver injury which adds up to another mechanism of liver induced injury caused by the virus. Another important consideration in the pathogenesis of liver dysfunction in patients with COVID-19 is the myriad of drugs that have been tried and are currently being used for treatment that cause hepatic injury *via* hepatocellular damage and cholestasis[140,141]. Reactivation of hepatitis B is associated with use of biological agents such as tocilizumab which has been used and studied in the treatment of COVID-19[142]. Thus a multitude of factors including inflammatory mediated damage, direct cytotoxicity, hypoxemia/hypotension mediated ischemic injury and drug induced injury contribute to the pathogenesis of hepatic damage in COVID-19. Figure 2 depicts the multiple factors contributing to hepatic injury in COVID-19.

The pattern of liver injury seen in COVID-19 is typically elevated AST and ALT levels with a predominance of AST elevation[143]. Serum bilirubin levels can also be mildly increased but it's relation to disease severity is unclear in contrast the levels of aminotransferases that correlate with disease severity[144]. Hypoalbuminemia can also be seen along with increased levels of GGT in severe cases, but the levels of ALP are usually normal in mild or severe cases[139,145]. A case report of a patient initially presenting with hepatitis that was later diagnosed as COVID-19 infection, has also been reported[146]. Patients with pre-existing liver disease may be more susceptible to suffer from liver damage from COVID-19 according to the meta-analysis done by Mantovani *et al*[138]. Patients with non-alcoholic fatty liver disease (NAFLD) were demonstrated to have higher risk of disease progression, longer viral shedding time and higher likelihood of abnormal liver function tests[147]. Table 3 describes relevant studies in the context of COVID-19 and liver disease[148-157].

Table 3 Studies studying coronavirus disease 2019 infection and liver disease

Ref.	Patients	Type	Study highlight with regards to liver disease
Xie *et al* [148]	79	Retrospective study	Liver injury maybe related to systemic inflammation and liver function should be monitored in patients with severe pulmonary lesions on imaging
Zhang *et al*[149]	115	Retrospective study	Liver enzymes as well as INR significantly elevated in patients with severe COVID-19; Albumin low in severe cases
Huang *et al*[145]	41	Prospective case series	Two percent patients had chronic liver disease; 37% patients had elevated AST which was more pronounced in ICU patients
Fan *et al* [141]	148	Retrospective case series	In patients with abnormal liver function, more received treatment with lopinavir/ritonavir as compared to those with normal liver function
Wang *et al* [150]	138	Retrospective study	Of 2.9% patients had chronic liver disease, AST elevation > ALT and seen more in ICU patients
Xu *et al* [151]	62	Retrospective study	Of 12% patients had underlying liver disease; 16% patients had elevated AST
Shi *et al* [152]	81	Retrospective study	AST more elevated in patients with increasing pulmonary lesions on imaging; 9% patients had hepatitis or cirrhosis on imaging
Zhang *et al*[153]	82	Retrospective study; Jul 2020	Of 2.4% patients had underlying liver disease; 1.2% patients died due to liver disease; 30.6%, 61.1% and 30.6% had elevated levels of ALT, AST and Total bilirubin respectively
Guan *et al* [135]	1099	Retrospective study	There are 2.1% patients had hepatitis B; AST, ALT and Total bilirubin were elevated in 22.2%, 21.3% and 10.5% patients respectively
Ji *et al* [147]	202	Retrospective study	Liver injury frequent but mild in nature with mostly hepatocellular pattern; Patients with NAFLD and BMI had higher risk for persistent liver injury. Patients with NAFLD had higher risk for severe COVID-19 and longer viral shedding.
Mao *et al* [154]	6686	Systematic Review and Meta-analysis	Pooled prevalence of liver comorbidities was 3%. Pooled prevalence of liver injury was 19%; Patients with severe COVID-19 had higher risk for abnormal liver enzymes.
Singh *et al* [155]	2780	Multicenter research network study	Patients with cirrhosis and pre-existing liver disease are at increased risk for hospitalization and death
Bloom *et al*[143]	60	Prospective cohort study	Predominant AST elevation commonly seen in COVID-19 and correlates with disease severity
Wang *et al* [156]	105	Retrospective study	Elevated liver enzymes more likely in patients with severe COVID-19
Cai *et al* [157]	417	Cross sectional study	Of 76.3% patients had abnormal liver enzymes and 21.5% had liver injury during hospitalization; Patients who received lopinavir/ritonavir had higher odds of liver injury. Patients with abnormal liver tests had higher chance of severe COVID-19

INR: International normalized ratio; COVID-19: Coronavirus disease-2019; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ICU: Intensive Care Unit; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

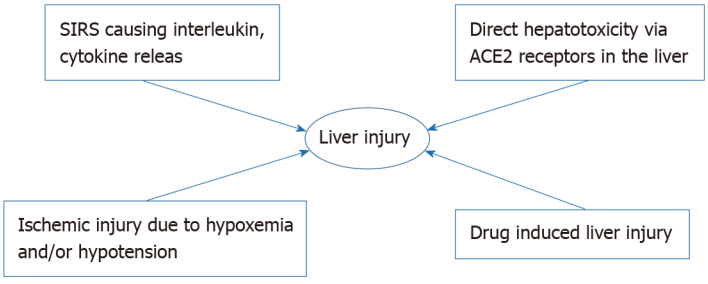


Figure 2 Multiple factors contributing to hepatic injury in coronavirus disease 2019.

COVID-19 in LT recipients

The risk of COVID-19 infection and its severity remain unclear in patients with LT, although a preliminary analysis of the SOT recipient registry from the University of Washington reported that the risk of contracting COVID-19 in SOT recipients is comparable to the general population[158]. With regards to mortality in LT patients, older patients with LT seem to have a higher mortality[159]. International voluntary registries that collect information on COVID-19 patients with underlying liver disease

Table 4 Studies evaluation coronavirus disease 2019 infection post liver transplantation

Ref.	Patients	Type	Study highlight with regards to liver transplant
Coll *et al*[168]	110	Retrospective	Higher incidence of COVID (two-fold) in solid organ transplant patients. Eighty-five percent patients had adjustment in their immunosuppression
Becchetti *et al*[169]	57	Multicenter Prospective	Of 12% overall fatality rate and 17% in-hospital fatality rate. Patients with history of cancer had poorer outcomes
Colmonero *et al*[170]	111	Prospective	LT patients with increased risk of contracting COVID-19 but lower mortality when compared with matched general population. Dose reduction/withdrawal in mycophenolate helped prevent severe COVID-19 but complete discontinuation of immunosuppressants discouraged.
Webb *et al*[171]	151	Multicenter Prospective	Need for invasive mechanical ventilation and ICU admission more in LT group when compared with a control cohort – 20% *vs* 5 % and 28% *vs* 8% respectively. LT not independently associated with death, but presence of comorbidities and increased age were
Belli *et al*[172]	240	Multicenter retrospective	Of 84% patients required hospitalization, 25% of hospitalized patients died. Use of Tacrolimus associated with increased survival probability
Bhoori *et al*[159]	111	Retrospective	Three patients died of COVID-19 and all of them were male, > 65 years with multiple comorbidities and minimal immunosuppression
Rabiee *et al*[173]	112	Prospective	Hospital and ICU mortality rates lower rates in matched patients with chronic liver disease without LT
Mansoor *et al*[174]	126	Retrospective	Higher risk of hospitalization in LT patients. No difference in mortality and need for ICU in LT patients *vs* non- LT patients
Tejedor-Tejada *et al*[175]	16	Retrospective	Post COVID-19 syndrome present with mild symptoms but no loss of liver graft or graft dysfunction noted

LT: Liver transplant; ICU: Intensive care unit; COVID-19: Coronavirus disease-2019.

and LT described 81% patients hospitalized with 30% requiring intensive care unit care and 19% expired[160]. A systematic review described a case fatality rate of 37.5% among LT recipients[161].

Organ procurement has decreased due to the limitations of the pandemic whereas telemedicine is increasingly utilized in evaluation LT recipients[162,163]. All the major societies recommend that patients with high MELD scores, risk for decompensation or HCC progression only be considered for LT[162,164,165]. AASLD recommends that patients with COVID-19 do not receive LT but the procedure can be undertaken 21 d after symptom resolution and negative test in recipient. With regards to immunosuppression in the post-transplant period, all the major societies recommend against reducing it as there has been no data to suggest immunosuppression as a risk factor for severe COVID-19[162,164,165]. AASLD however recommends lowering antimetabolite medication dosages while maintaining the same doses of calcineurin inhibitors (CNI) in LT patients with COVID-19, based on similar principles for managing an active infection in LT patients. Managing immunosuppressive therapy is challenging and should be done cautiously in patients with LT who had COVID-19 due to interactions between corticosteroids and CNI, and the liver toxicity associated with remdesevir and tocilizumab[166]. AASLD recommends vaccination preferable 3 mo after liver transplant once the doses of immunosuppressant medications have been reduced[167]. Table 4 highlights important studies in the context of COVID-19 and liver transplant[168-175].

CONCLUSION

The topic of non-hepatotropic viral infection is very broad and covers a number of infections that do not have liver as the primary site of infection. Majority of the known infections belong to the family of herpes virus infections and often require reactivation, as seen in immunocompromised individuals. Since the development of systemic disease with these infections depends on immune dysregulation, full blown disease is rarely seen in immunocompetent patients. Moreover, the infection is more severe in immunocompromised individuals, especially post-transplant (including liver transplant). These patients can also suffer from allograft rejection, in addition to hepatitis of varying degree of severity. The diagnosis, despite the presence of new testing modalities, is often based on exclusion of hepatotropic infection and liver

biopsy findings inconsistent with other etiologies of hepatitis. There is a lack of guidelines regarding management of each viral infection. Antivirals are often the first line, with or without steroid use. Patients with poor prognosis are worked up for liver transplant and studies have indicated continued use of antivirals following transplant to cover for latent infection. Lastly, there is growing literation on the involvement of liver in coronavirus 2019 pandemic and warrants it to be included in the differential diagnosis of hepatitis, once hepatotropic infection is ruled out.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 543-556

DOI: 10.13105/wjma.v9.i6.543

ISSN 2308-3840 (online)

MINIREVIEWS

Prediabetes and cardiovascular complications study: Highlights on gestational diabetes, self-management and primary health care

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Abstract

International collaboration on the prediabetes and cardiovascular complications study started in 2013. In 2017, a reflection was reported. Incompleteness of documentation and screening of antenatal cases for gestational diabetes mellitus

Okuleye A and Ezugwu EC were involved in discussions throughout the study and made intellectual contributions to the study performance; Nwose EU, Bwititi PT and Agofure O drafted the manuscript; All authors revised the manuscript and approved the final version for submission.

Conflict-of-interest statement: All authors have no conflict-of-interest to declare.

Supported by Bringing Research in Diabetes to Global Environments and Systems (BRIDGES2) program.

Country/Territory of origin: Nigeria

Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

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Received: January 28, 2021
Peer-review started: January 28, 2021
First decision: May 3, 2021
Revised: May 21, 2021
Accepted: November 28, 2021
Article in press: November 28, 2021

(GDM) was concerning. Hence, further observations have been made that warrant an update. The objective of this review is to highlight gaps between clinical knowledge and practice in GDM, diabetes self-management and primary health care (PHC) for rural dwellers. We followed a descriptive field notes method. Antenatal records of patients screened for GDM with incomplete documentation were examined to determine incompleteness of data in those that also met the criteria for GDM risk assessment. Experiences on development of a diabetes register and education and notes on behavioural change wheel were also reviewed. Other data included cross-sectional evaluation of activities of daily living at two private hospitals. Up to 29% had high GDM risk factors, which fulfilled selection criteria for laboratory screening. Demographic data was complete in all women; however, incomplete documentation was observed with as much as 98% of basic data. High levels of physical activity were found in the population, and health lectures proved effective in food choices. The workforce need for diabetes care seems underestimated, but this may be better understood with reactivation of PHC services. The observations highlight behavioural change wheel issues on GDM and PHC services that need concerted focus. Two proposals are to advance the use of a 'risk assessment and screening sheet' for GDM screening and enlightenment of stakeholders on the central hub role of PHC in diabetes management.

Key Words: Behavioural change wheel; Community health; Diabetes education; Knowledge *vs* practice gap; Lifestyle; Patient follow-up; Screening services; Telehealth

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Core Tip: There is a gap between knowledge and practice among stakeholders in diabetes management. Behavioural change wheel issues on gestational diabetes mellitus (GDM) and primary health care (PHC) services need concerted focus. The necessity for GDM selective screening is recognized. 'Risk assessment and screening sheet' needs to be employed in clinical practice. Stakeholders including individuals living with diabetes, community leadership, policy makers and health care provision staff need enlightenment to deliberately use PHC centres for diabetes management. PHC centres have a central-hub role in community health. Medical records need to include patients' phone numbers to maximize potential for follow-up and telehealth.

Citation: Nwose EU, Bwititi PT, Agofure O, Oshionwu EJ, Young EE, Aganbi E, Egwenu SE, Chime HE, Gbeinbo FD, Odufu A, Okuzor JN, Okuleye A, Aninze K, Onyia IC, Ezugwu EC, Igumbor EO, Ulasi II. Prediabetes and cardiovascular complications study: Highlights on gestational diabetes, self-management and primary health care. *World J Meta-Anal* 2021; 9(6): 543-556
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/543.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.543

INTRODUCTION

Diabetes mellitus is a global challenge and public health concern that has continued to attract multifaceted research[1-3]. These interests include laboratory examinations for definitive diagnosis of gestational diabetes mellitus (GDM)[4-6], lifestyle treatment options[7-9] and use of technologies for patient follow-up and self-management[10-12].

In 2013, a proposal for an international collaboration on prediabetes and cardiovascular complications study was launched[13]. In 2017, an update with future directions was reported[14]. In the last 3 years, the program has been expounded, with niches developing from parts of the research. The Global Medical Research and Development Organization that oversees the activities at Catholic Hospital Abbi conceptualized the integration of a monthly diabetes clinic and GDM screening and registry for antenatal clinic patients[15-17]. A user-friendly GDM risk assessment and

Published online: December 28, 2021

P-Reviewer: Pustozerov EA
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

screening form was proposed in January 2020 in collaboration with the University of Nigeria Teaching Hospital.

An opportunity arose after the acquisition of the BRIDGES2 grant [Eli Lily and International Diabetes Federation (IDF)] for a targeted diabetes self-management education trial. This created impetus to develop the lifestyle component of the research. In addition to reports[18-20], two field notes detailing dietary options and food choices as well as physical activities of daily living (ADL) were presented.

To further add to the diabetes registry and BRIDGES2 project, the Global Medical Research and Development Organization reviewed primary health care (PHC) services. This included a discussion about telehealth capacities to enhance diabetes services[21,22]. Telehealth is becoming an invaluable resource. The lessons from the coronavirus disease 2019 pandemic have proven this. Hence, field note observations relevant to PHC and telehealth constitute part of this update, including lessons from the World Diabetes Day (WDD) 2020.

Objective and approach of review

The objective of this paper is to give a periodic review of trends[23-25]. This review is based on our current prediabetes and cardiovascular complications study research with a focus on the gap between knowledge and practice in laboratory testing for GDM diagnosis, including the use of risk assessment factors for selective screening. Other focus areas are emerging treatments (specifically nutrition) and technologies (medical record documentation and telehealth).

DESCRIPTIVE FIELD NOTES REVIEW

This review was designed to follow a descriptive field notes study method. The notes were based on the international collaboration on a prediabetes and cardiovascular complications study program. The study was carried out in Delta State, Nigeria and at health care centres/hospitals. For this update, the 'project evaluation' looked at notes and data from the ongoing study, and the goals for each section are given as subheadings.

GDM screening

GDM constitutes a subsequent health risk to the mother and child. Several risk factors for GDM are known; hence, screening is encouraged. However, the adoption of robust screening protocols in various health care facilities is lacking, and reports have indicated a similar situation in our research performance sites[15,29]. One notable observation was that selection criteria for lab tests in GDM screening were being followed[16] but arbitrarily and with rampant incompleteness of procedure. Therefore, while a screening flow sheet has been articulated[15], it was appropriate to develop another sheet with selective screening criteria (Table 1). This proposed GDM risk assessment and screening sheet was informed by two points: (1) Risk factors are generally non-invasive and can be obtained during antenatal registration and history taking[30-32]; and (2) Risk scoring systems exist. One includes gestational age[31], while the other is without gestational age[32].

Standard selection for screening: A user-friendly algorithm or chart is yet to be developed. Pending the adoption of the risk scoring chart, this evaluation would assess antenatal patients with three risk factors for laboratory investigation of GDM. Two retrospective cohort reviews have tabulated the GDM risk assessment and screening sheet (Table 1). Furthermore, the mother's blood type is a potential genetic factor for the likelihood of GDM developing overt diabetes mellitus (DM) postpartum [33]. Blood type assessments are determined for antenatal patients but may not be included in GDM screening sheets. Nevertheless, it is important to identify this as part of GDM management.

GDM screening register: In another field note, evaluation was specifically aimed to (1) Progress the development of screening registers, (2) Determine the level of attention given to or the potential of monitoring the rate of increase in body mass index (BMI) among the patients; and (3) Update the prevalence of GDM among pregnant women.

Diabetes self-management

Four items are imperative components of behavioural change included in diabetes education programs[34]. They are dietary options, physical ADL, adherence to medical

Table 1 Proposed gestational diabetes risk assessment and screening sheet

SN	Assessment stage	Factor	Yes	No
1	Initial routine data	Age > 35 yr		
2		Overweight[1] (BMI > 25)		
3		Hypertension		
4		Sedentary lifestyle[2]		
5		Family history of diabetes		
6	Previous history	GDM		
7		Miscarriage		
8		Foetal/neonatal death		
9		Polycystic ovary syndrome		
10	Antenatal monitoring	Presentation of GDM symptoms		
11		Excess gestational weight gain[3]		
12		Foetal growth		
13		Macrosomia		
14	Lab tests[5]	Urinalysis for glucosuria		
15		Blood sugar test[4]		

Keys to positive risk factors for 'Yes' response.
[1]Especially obesity (body mass index > 30), which also defines nutritional status.
[2]Activities of daily living being non-physical and/or mainly sitting position.
[3]> 1.80 kg in first trimester and/or > 0.45 kg per week during the rest of pregnancy.
[4]Interpret according to method.
[5]For antenatal GDM screening.
GDM: Gestational diabetes.

check-ups and adherence to prescription medicine. For this update, two field notes on dietary options and physical ADL are presented.

Physical ADL: Physical inactivity predisposes a person to DM. Thus, engaging in physical activities is preventive and therapeutic. The study evaluated ADL of local residents with diabetes who attended two non-government health facilities in rural communities of Delta State, Nigeria. This study was an addendum to a previous report [20]. A cross-sectional descriptive design was used and comprised 48 participants from Catholic Hospital Abbi and Novena Health Centre Amai. A semi-structured questionnaire with integrated anthropometric and blood pressure measurements (weight, height, blood pressure, and BMI) was used. ADL of participants was delineated into blue *vs* white collar jobs, depicting physically-active and -inactive occupations respectively. The data were analysed using the Microsoft Excel Analysis ToolPak. A demographic description of the respondents is presented in Table 2. Among the women, 11/21 (52.4%) were widows, and the average age of all respondents was 57 years.

Dietary options and food choices: In another study niche, the consumption of indigenous carbohydrate foods was evaluated. This niche was originally studied to determine the agribusiness and health economics nexus[35], but the basis of this field note was the controversy around cassava[36,37] that was buoyed by indigenously sourced cassava meal (eba/garri) being omitted from a hospital-based menu[19]. The study involved baseline and post-intervention surveys of community members including hospital visitors, and the intervention was public health lectures on medical nutrition therapy value of cassava. Information collected included consumption habits regarding farmed choices (cassava and yam) and an imported option (wheat).

Other evaluations

PHC needs – lessons from WDD 2020: With the invaluable support from the Public Health Department of Novena University, the WDD event was organized at 11 health

Table 2 Descriptive statistics of respondents

		Male	Female
	n	**27**	**21**
	Age in yr	59	55
Educational status	≤ Secondary school	19	14
	≥ Tertiary education	8	7
Marital status	Married	23	10
	Single	4	11

facilities, including four research performance sites, a general hospital, two PHC centres in the Delta state, one centre in the southern Nigeria, and three centres in northern Nigeria. There were 23 volunteers comprised of academic staff and postgraduate students of the Public Health Department of the Novena University who facilitated the event. An additional 79 health care personnel were recruited who delivered public health lectures and diabetes screening. A total of 286 community members benefited from the event[38]. Observations were made from this event.

Telehealth potential in rural communities of Nigeria: Development of diabetes registers was started at the research performance sites, *i.e.* health facilities. However, evaluation of the usefulness for patient recall and telehealth have yet to be reviewed. Hence, in the hospital cohort study of 123 cases, the evaluation approach for telehealth involved assessing previous antenatal patients by telephone.

PERSPECTIVES ON GDM, SELF-MANAGEMENT AND PHC IN PREDIABETES AND CARDIOVASCULAR COMPLICATIONS

GDM screening

In a hospital cohort involving 90 cases, there was no urinalysis on the antenatal patients despite various prevalent risk factors. A history of GDM was the least common risk factor, and age over 35 years was the most common risk factor (Figure 1). Considering the prevalence of risk factors at initial registration, the lack of urinalysis is inexplicable. In another hospital cohort of 123 cases, 29% of those assessed had three or more risk factors that warranted screening (unpublished data). These reports provide evidence that screening education should be provided to antenatal providers.

Level of completeness of data collected during GDM screening: We collected data from 391 antenatal cases that were seen from September 2018 to December 2018. The women's ages ranged from 15 to 46 years, and average gestational age was 23 wk, with the earliest clinic visit occurring at the fourth week. Further, several of the women were in their first pregnancy, and cohort average was less than gravida 3 (Table 3).

Among the 391 cases, the names, ages, medical record numbers and dates of hospital visits were complete, *i.e.*, 100% documentation. Information on the mother's and/or family history of type 2 diabetes and GDM were not indicated, *i.e.*, 0% documentation on hereditary risk factor. There were also no records of lipid profiles. Other relevant data that were collected are presented in Table 4. Blood sugar tests were undocumented for 98.7% of patients, and BMI was not reported for 76.7% of patients. Overall, our results show that the average level of completeness of data collection during GDM screening was 79% (Figure 2). The figure shows the percentages of completeness that were not captured in Table 4.

Level of attention given to BMI: The 301/391 cases lacking BMI measurement had missing data on height (Table 4), but only 14 Lacked weight measurements. A critical evaluation showed that 5% of the cases without BMI values were due to lack of height and weight measurements, and 95% were solely due to lack of height data (Figure 3). Further review of the potential monitoring rate of increase in BMI among the patients showed that 13% (12/90) were in the first trimester and 48% (43/90) in the second trimester. There was a noticeably higher level of BMI in the third trimester relative to the earlier trimesters, but the difference was not statistically significant.

Table 3 Descriptive statistics of patients entered in a gestational diabetes screening register

	Age in yr	Gestational age in wk	Gravida[1]
Mean	28.57	23.01	2.60
Standard error	0.30	0.41	0.08
Median	28.00	23.00	2.00
Mode	25.00	24.00	1.00
Standard deviation	5.91	7.33	1.63
Sample variance	34.97	53.72	2.67
Range	31	35	9
Minimum	15	4	1
Maximum	46	39	10
Count	391	324	377

[1]Number of times a woman has been pregnant.

Table 4 Level of incompleteness of data

Missing data	*n*/391	Absolute Hz	Relative Hz
Address	1	0.2558	0.0760
Rh factor	6	1.5345	0.4563
HVS, 2/385 positive	6	1.5345	0.4563
Urine glucose	6	1.5345	0.4563
ABO	8	2.0460	0.6084
Marital status	12	3.0691	0.9125
Weight in kg	14	3.5806	1.0646
SBP in mmHg	14	3.5806	1.0646
DBP in mmHg	14	3.5806	1.0646
Gravida	14	3.5806	1.0646
Packed cell volume, %	15	3.8363	1.1407
Phone number	60	15.3453	4.5627
Gestational age in wk	67	17.1355	5.0951
Occupation	92	23.5294	6.9962
Height in cm	301	76.9821	22.8897
BMI	301	76.9821	22.8897
Blood sugar, as FBS or RBS	386	98.7212	29.3536

ABO: Blood grouping system; BMI: Body mass index; DBP: Diastolic blood pressure; FBS: Fasting blood sugar; HVS: High vaginal swab; RBS: Random blood sugar; SBP: Systolic blood pressure.

ADL in the community

This analysis was based on the occupation of the participants. The indicated occupations were categorized into blue collar (manual labour, *e.g.*, agriculture, construction, *etc.*) and white collar (office or non-hard manual work professional) depending on assumptions of physical demands of the jobs. Forty-eight respondents were analysed, including four who did not indicate their occupations and were assumed to be blue collar. Evaluation showed that 73% were involved in physically demanding ADL (Figure 4).

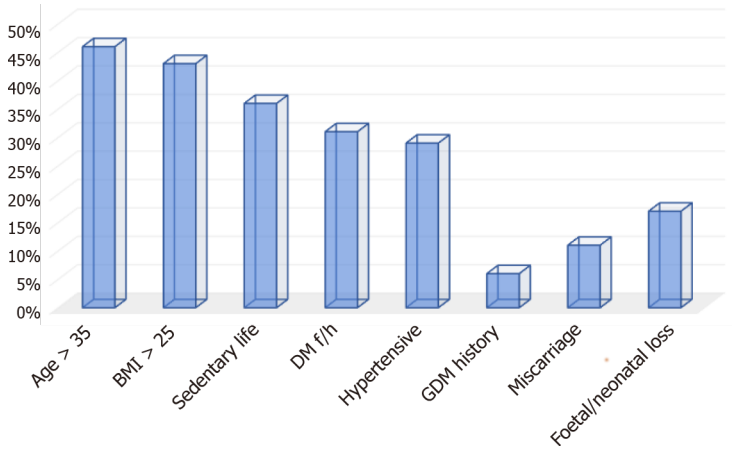


Figure 1 Prevalence of gestational diabetes risk factors in a hospital cohort from Delta State, Nigeria. History of gestational diabetes mellitus was the least common risk factor, while age was the most common risk factor. BMI: Body mass index; DM: Diabetes mellitus; GDM: Gestational diabetes mellitus.

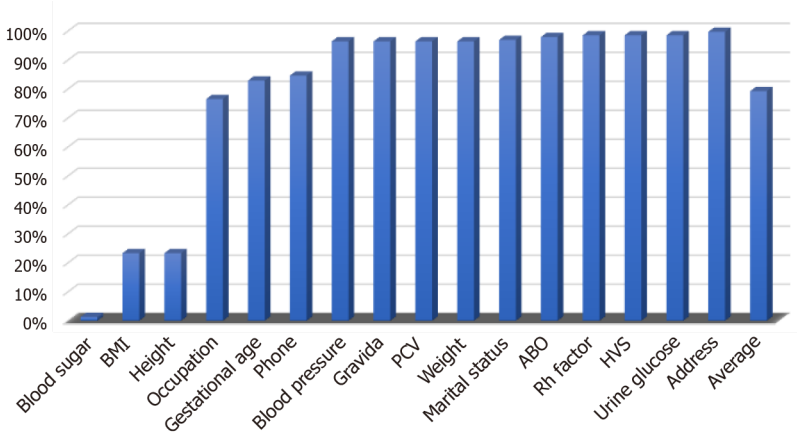


Figure 2 Translated percentage completeness of data collection. Most antenatal patients lacked blood sugar and body mass index measurements. ABO: Blood grouping system; HVS: High vaginal swab; PCV: Packed cell volume; BMI: Body mass index.

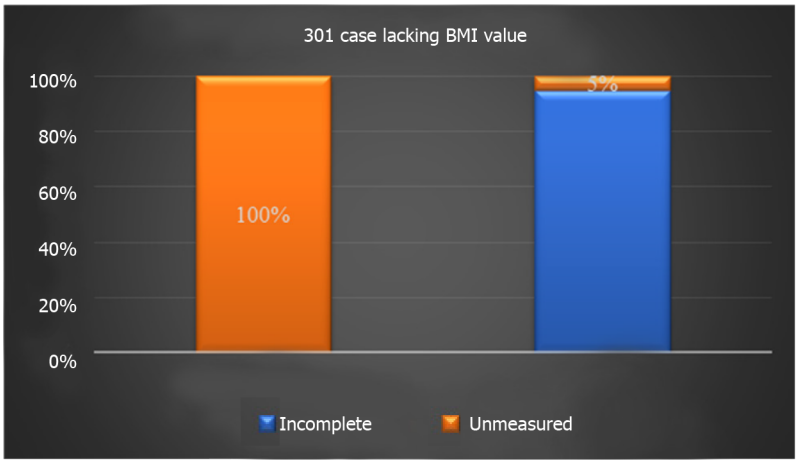


Figure 3 Proportions of missing body mass index values attributable to either missing height or weight. Most cases without body mass index data were due to a lack of height measurement. BMI: Body mass index.

Level of physical activities among participants

Evaluation of responses to exercise ADL indicated that over 50% of the respondents were not able to stretch or swim, while walking appeared to be the activity for most (Table 5). Evaluation of interference of basic ADL showed that ill health interfered

Table 5 Responses to exercise abilities, *n* = 48

Duration	Stretching	Walking	Swimming	Bicycling
None	28	15	41	24
< 30 min	11	10	2	6
30 min–1 h	4	13	3	7
1–3 h	2	6	2	4
> 3 h	3	4	0	7

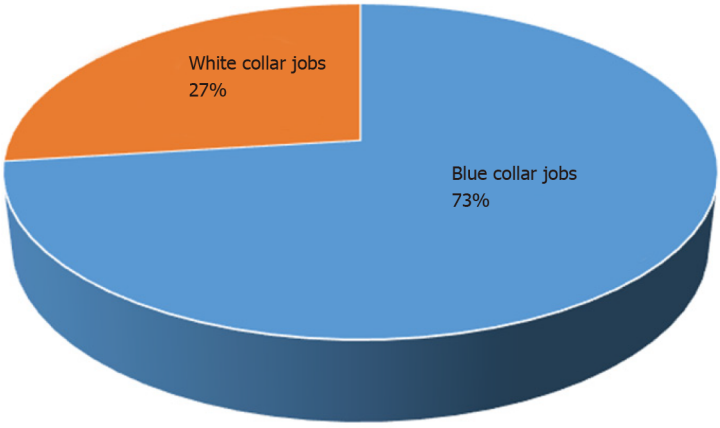


Figure 4 Distribution between manual and non-manual jobs. Most cases were in occupations with physically demanding activities of daily living.

mostly with respondents' hobbies, but generally all people engaged in a variety of physical activities.

Effect of diabetes on the ADL of participants in the community

When participants were categorized based on DM and hypertension + DM, the results showed that diabetes interfered with hobbies and work the most. However, the interference was minimal. The impact of hypertension + DM on all the surveyed ADL items was higher (moderate interference of hobbies and work) (Figure 5).

It was concluded that participants in manual jobs constituted about three-quarters of the diabetes patients. Nevertheless, the proportion of those in non-manual jobs with hypertension comorbidity was higher, which implies healthy occupational ADL as indicated by a preponderance of manual occupations in people with diabetes. The impact of ill health on leisure ADL was also indicated, especially that it was worse in those with hypertension comorbidity. This highlights that a pivotal focus on diabetes self-management regarding physical activities should include evaluation of the patient's capacity to engage in ADL, especially as the impact of diabetes on ADL translates to a barrier of a therapeutic lifestyle regimen. Given that the interference of ADL by ill health was worse among those with hypertension and diabetes, both groups require different levels of care.

Effect of public education on food consumption

A baseline survey showed that 100% of rural farmers harvest cassava. The survey also showed that only 48% consumed cassava as one of their food choices, and 10% consumed wheat only and disregarded other options (Figure 6). This observation differed from other cohorts, such as urban dwellers who were wheat consumers and included a high proportion of educated people. After the public health lecture, the post-intervention survey showed a shift in consumption patterns, in favour of cassava products[39].

WDD 2020 highlights

At WDD 2020, the IDF highlighted the following three urgent facts about Nigeria and diabetes: (1) Six million nurses are needed; (2) About 50% of adults living with diabetes are undiagnosed; and (3) Over 75% of people living with diabetes live in

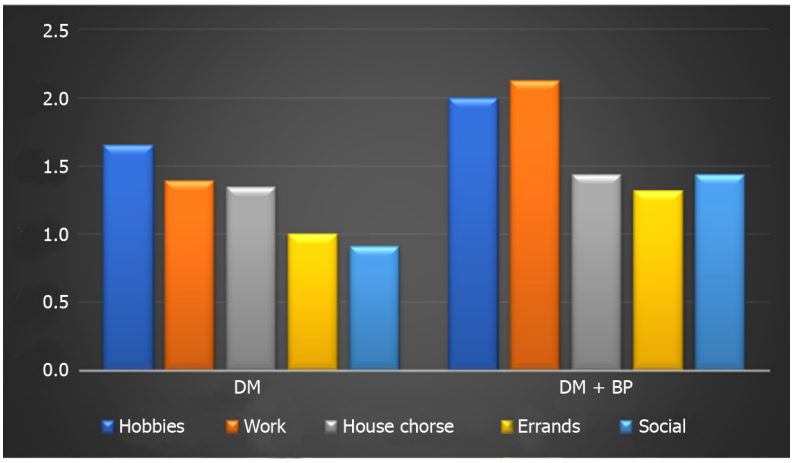


Figure 5 Average responses on interference of ill health on activities of daily living. The group with diabetes mellitus only was more responsive to physical activities of daily living. DM: Diabetes mellitus; BP: Blood pressure (indicated hypertension in this graph).

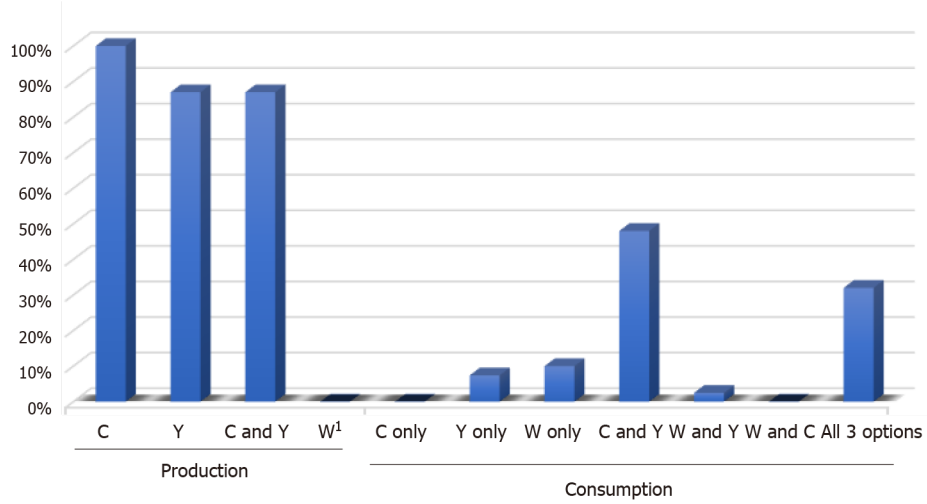


Figure 6 Consumption and production carbohydrate food choices. Most individuals undervalued their indigenous healthy food options, such as cassava and yams. [1]Never farmed in the area. C: Cassava; W: Wheat; Y: Yam.

urban areas and are of working age.

Lessons from the event can be summarized in two themes. First, there is an underestimated burden of diabetes in terms of the nursing workforce requirement in PHC. During preparation for the event, it was determined that the 6 million projection is an underestimation. This determination was based on: (1)Australia being speculated to require about 120000 nurses in the next 10 years, which is 2% of the projected 6 million need[40]; and (2) More than 75% of people in African countries being undiagnosed for diabetes[41], making the actual burden yet to be estimated. This implies that Nigeria, with a population of over 200 million, needs to train and recruit many health care workers in diabetes care. Considering the current nursing workforce in the country, Nigeria will need more than the estimated 6 million.

Non-governmental organizations run diabetes screening services at community and PHC centres, but resident staff of the health care centres do not. Therefore, the knowledge is present but is not practiced, hence the apparent gap in knowledge *vs* practice. Importantly, most of the health care workers in the rural community-based PHC facilities are unaware of the WDD diabetes educator courses. However, these courses are well-received because the general hospitals requested a reschedule of the WDD from a Saturday to a weekday, in order for the staff to benefit. In addition, at three of the hospitals, the event was carried out twice, once for the general public and again for the hospital staff.

Telehealth

Our data indicates that 20% of the cases on medical records did not have phone numbers on record. However, 80% of the patients were able to receive services *via* telehealth. A pertinent observation is that some of the phone numbers were actually owned by the patient's child who was not living with the patient. Although many of the calls were unsuccessful, 34% of the cohort received telehealth services, and 29% of the sub-cohort received health advice (Figure 7). In light of the coronavirus disease 2019 pandemic and its effects on routine health care provision, this is an area that can be further utilised and expanded.

DISCUSSION

This review highlighted some of the problems in diabetes research and practice. Specifically, the issues of GDM, nutrition in terms of carbohydrate food choices, PHC, and telehealth. Links have been made to the concept of behavioural change wheel (BCW_ regarding the gap between knowledge *vs* practice. BCW is important in managing chronic conditions. It is a guide for designing intervention programs, and it is predicated on capacity, motivation, and opportunity[26]. It is a comprehensive framework for designing interventions by explicitly integrating behaviour theory to understand and target mechanisms of action within the intervention[27], and it is mindful that individual and collective behaviour change is key to implementing new practices and to improving health outcomes. It also incorporates context, which is key to effective design and implementation of interventions[28].

There has been a lack of clarity on the universal screening of pregnant women for GDM[42-44], and the result is that this service remains under-utilised[45]. This low level of GDM screening service appears to represent a gap between knowledge and practice among the clinicians. The positive note is that GDM risk assessment for selective screening of pregnant women now exists[30-32]. The user-friendly risk assessment sheet needs to be validated for local clinical use and integrated into GDM screening services. Two notable pieces of clinical knowledge include (1) BMI may be more reliable as a risk factor when measured in the first trimester, and (2) More weight measurements should be done because gestational weight gain is imperative in monitoring well-being of the baby and mother. Therefore, most antenatal clinics assess these at every visit, but height is documented at registration. This review highlighted the proportions of cases missing different necessary measurements and/or documentation.

Our cohort of 391 antenatal patients in the register were reviewed to determine the number of conclusive GDM diagnoses. This review was an addendum to a previous observation[15], and it was observed that 385 patients had a urine glucose test, out of which 16 (4%) were positive for glucosuria. Glucosuria may be found in about 50% at some point because of the associated changes in glomerular filtration rate. However, a follow-up blood sugar test for at-risk women is still essential[46]. None of the 16 cases were followed-up with a blood sugar test. Instead, 5 other patients had blood glucose test results without urinalysis. Therefore, we do not know the incidence rate of GDM at our hospital. This highlights a knowledge and practice gap in antenatal patient laboratory test requisition and follow-up.

The four components of necessary diabetes education are: Adherence to medication, nutritional lifestyle, physical ADL, and up-to-date medical check-ups including laboratory tests[2,47,48]. This review reported on carbohydrate nutrition and highlighted that people are still unaware of the values of their indigenous foods. This lack of knowledge was identified long ago[49], hence this report suggests that the problem is yet to be resolved. Therefore, more concerted efforts are needed to advance the knowledge of the glycaemic index of Nigerian carbohydrate foods. This report also highlighted physical ADL affordances and their effectiveness in the rural community.

PHC care is a function of the state, regarding the government's policy and practice on health services. One of the issues, at least in developing communities such as Africa, is limited resources[50]. However, studies on knowledge, attitude and practice have indicated that there are patient factors to consider[51]. The Alma Ata declaration identified the necessity and potential of PHC to play a central hub role, and diabetes care services are encompassed[52]. This report highlights a known problem that is still unresolved.

Therefore, it is recommended for the World Health Organization and other stakeholders (community, community leadership, policy makers and health care provision staff) to focus on PHC for diabetes care. For instance, looking at the Nigerian

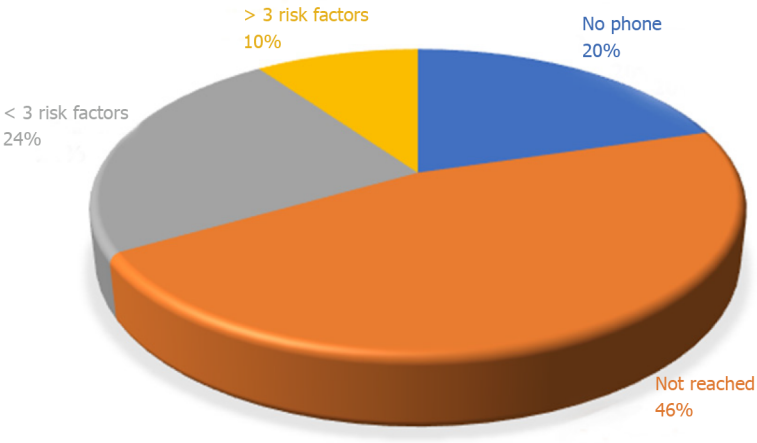


Figure 7 Distribution of cohort of cases assessed by telehealth. Most cases provided incomplete or incorrect phone numbers on their medical records.

health care system, there are PHC centres scattered and operated by local governments, but the community dwellers rarely patronize the centres. By implication, the feedback to the government is hampered, and the impression is that "the government is not doing enough." However, it is not entirely the fault of the government *per se*[18]. This requires behavioural change of the community, inclusive of the ministry of health[53]. It was reported from a BCW survey that about 40% of stakeholders agreed on the ability of PHC to offer diabetes services, whereas over 70% believed the motivation was poor[18]. Therefore, this update highlights one area of need of BCW for IDF and the World Health Organization.

Over the past 3 years, translational research efforts have included development of a Diabetes Registry in the health facilities[17,54,55]. It has been reported that a major factor to consider is concerted integration of telephone numbers of patients in medical files[54]. For this obvious reason, the Global Medical Research and Development Organization is developing telehealth services for the area, of which telephone numbers are indispensable.

There is ongoing interest to utilise accessible and affordable telecommunication tools including telephones in community health service delivery[56-58]. This review reported the potential to use the telephone for follow-up in rural communities. It also highlighted two limitations, which are non-documentation of the patient's phone number or the patient providing another person's telephone. This observation calls for awareness for health care providers to document phone numbers in patients' records as well as alert patients on the importance of providing their own numbers.

CONCLUSION

These updated field note observations from ongoing research and topical research areas include prediabetes, diabetes registry, GDM screening concerns, PHC and telehealth potential for rural dwellers. From experience on the development of a diabetes register and intensive diabetes education, notes on BCW were reviewed and this is a work in progress, especially due to the negative impact of the coronavirus disease 2019 pandemic. Other field data included a cross-sectional study of ADL at two private hospitals. One of the important topics in diabetes care is that of PHC. There is a knowledge and practice gap for the important role that PHC must play in diabetes care, and this constitutes another point on BCW. This problem is likely confounded by the IDF and World Health Organization underestimating the nursing workforce. It is possible that a proactive PHC system equipped with various registries, such as a diabetes registry, could minimize the documentation issues as well as improving screening procedures. Mobile phones and internet services are becoming affordable and accessible, and research needs to focus on their utilisation. Future research should also advance BCW of all stakeholders (community, community leadership, policy makers and health care provision staff) to maximize the impact of diabetes research.

ACKNOWLEDGEMENTS

Some postgraduate and undergraduate students of the Public Health Department of Novena University supported the data collection during their research training, which have been duly published. This group includes Annette Orove, Edmond Anowa, Emmanuel Agege, Garba Ahmed, and Joseph Odoko. The staff and managements of Catholic Hospital Abbi, Donak Hospital Kwale, Eku Baptist Government Hospital, Friends Medical Laboratory Service and Novena University Health Centre are hereby appreciated.

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World J Meta-Anal 2021 December 28; 9(6): 557-567

DOI: 10.13105/wjma.v9.i6.557

ISSN 2308-3840 (online)

SYSTEMATIC REVIEWS

Hydroxychloroquine alone or in combination with azithromycin and corrected QT prolongation in COVID-19 patients: A systematic review

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Conflict-of-interest statement: The authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist statement, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist statement.

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Abstract

BACKGROUND

Despite the controversies about the effectiveness of the current drug regimens for coronavirus disease 2019 (COVID-19), these drugs are still the only options available. Moreover, the safety of these drugs is yet to be confirmed. A serious concern is the occurrence of various cardiac arrhythmias, particularly QT prolongation.

AIM

To summarize the incidence and estimate the risk of QT interval prolongation in patients scheduling for conventional treatment (hydroxychloroquine alone or in combination with azithromycin) for COVID-19.

METHODS

We comprehensively searched Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials databases until October 31, 2020 for all eligible studies under the considered keywords COVID-

Specialty type: Cardiac and cardiovascular systems

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Received: June 17, 2021
Peer-review started: June 17, 2021
First decision: July 30, 2021
Revised: August 1, 2021
Accepted: November 5, 2021
Article in press: November 5, 2021
Published online: December 28, 2021

P-Reviewer: Khan MKA
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The study protocols were established in compliance with PRISMA-P guidelines (Preferred Reporting Items for Systematic Review and Meta-Analysis – Protocols), and a nine-star Newcastle-Ottawa Scale scoring system was used to assess the methodological quality of all eligible studies. Outcome measures were corrected QT (QTc) prolongation, cardiac arrhythmias, or sudden cardiac death.

RESULTS

Fifteen studies enrolling 8298 patients with targeted COVID-19 therapeutic regimes were included. The eligible studies found a significant increase in the mean QTc interval following treatment with the described medications compared to baseline QTc with weighted standard differences in means of 0.766. The pooled prevalence rate of QTc prolongation was estimated to be 9.2% (95% confidence interval: 4.5% to 18.1%).

CONCLUSION

Hydroxychloroquine ± azithromycin regimen can significantly increase the risk of developing QTc prolongation.

Key Words: Azithromycin; COVID-19; Hydroxychloroquine; QTc interval

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Core Tip: Given the greater importance of coronavirus disease 2019 worldwide, there is an ongoing controversy about the potential harms of anti-viral agents in which caused uncertainties in daily clinical practice. Given the unresolved debate, during this systematic review and meta-analysis, we investigated the association of Hydroxychloroquine (alone or in combination with azithromycin) with the risk of QT interval prolongation, cardiac arrhythmias, and sudden cardiac death. Although there are some studies about the effects of these agents, there are scarce systematic reviews and meta-analyses about both QT prolongation and risk of cardiac arrhythmias which is a distinguishing point for our study.

Citation: Ashraf H, Ghafouri P, Kazemian S, Soleimani A, Sadat Naseri A, Karbalai S, Kazemi Saeid A. Hydroxychloroquine alone or in combination with azithromycin and corrected QT prolongation in COVID-19 patients: A systematic review. *World J Meta-Anal* 2021; 9(6): 557-567
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/557.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.557

INTRODUCTION

With the outbreak of the coronavirus disease 2019 (COVID-19) pandemic, we have seen high mortality rates from the disease in almost all countries involved[1]. Besides the pulmonary involvement in form of acute respiratory distress syndrome, one of the main features of this disease is the involvement of other systems, like cardiovascular, gastrointestinal, central nervous system, and even skin and mucosal involvement[2-4]. However, respiratory failure and subsequent cardiovascular compromise were major determinants for patients' survival[5]. So far, there are no successful and safe drug regimens for the treatment and prevention of COVID-19. Current approaches have either failed or have been withheld due to potential side effects. Thus, the side effects have added to the high mortality and morbidity caused by COVID-19[6].

Current evidence suggests that using hydroxychloroquine and azithromycin for COVID-19 increases the risk of cardiac arrhythmias[7,8]. Previous studies reported that these drugs caused corrected QT (QTc) prolongation, leading to life-threatening conditions like torsades de pointes (TdP) and sudden cardiac death[9,10]. Although both *in vivo* and *in vitro* studies recommended the combination therapy of azithromycin and hydroxychloroquine, even as the first-line approach in preventing disease, it has also led to QTc prolongation[11]. In addition to cardiac monitoring,

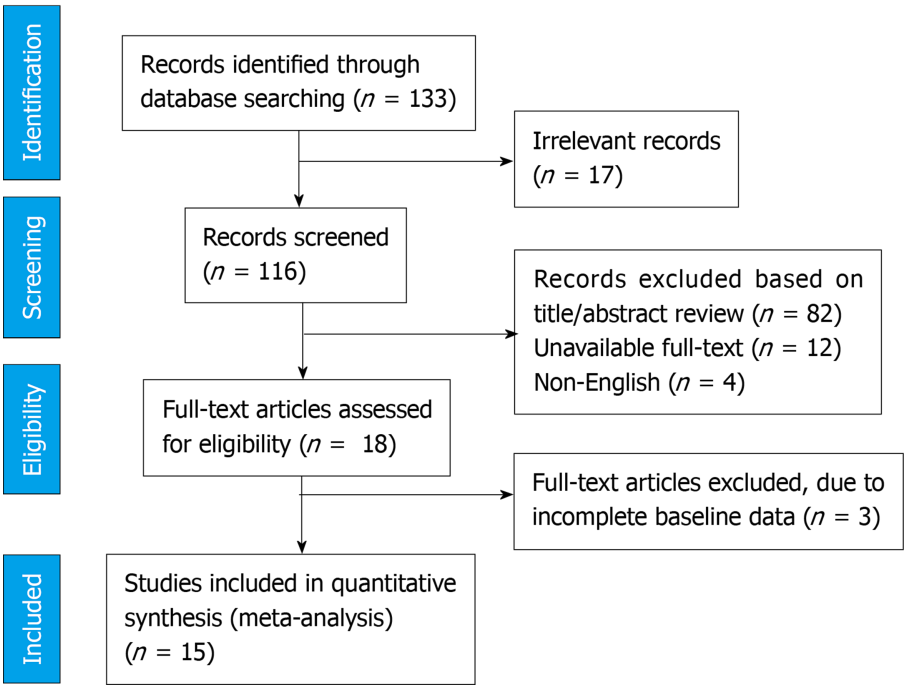


Figure 1 The flowchart of screening the eligible studies.

identifying patients, who are prone to the side effects, helps to minimize the potential harms. By identifying susceptible individuals, it may be possible to use other drug protocols to maintain patient survival. Herein, we summarize the findings about the prevalence and the risk of QTc prolongation in patients treated with hydroxychloroquine ± azithromycin. Also, we discuss the life-threatening conditions in patients taking these medications.

MATERIALS AND METHODS

Search strategy

We performed this review according to established methods and in compliance with PRISMA-P (Preferred Reporting Items for Systematic Review and Meta-Analysis) Protocols. Two investigators searched the manuscript databases including Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials in The Cochrane Library until October 31, 2020 for all eligible studies under the considered keywords including COVID-19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The studies were restricted to the English language. We included all randomized controlled trials, including individually randomized and cluster-randomized trials. We reviewed the studies reported as full-text and those published as abstracts. We also conducted a search of ClinicalTrials.gov and the World Health Organization International Clinical Trials Registry Platform Search Portal for ongoing or unpublished trials. The inclusion criterion was a study population of adult patients, who suffered from a definitive diagnosis of COVID-19 and were treated with at least one or a combination of these medications: hydroxychloroquine, chloroquine, and azithromycin. The exclusion criteria were: (1) A lack of clear and reproducible results; (2) Non-English studies; (3) Lack of access to the full texts of the articles; and (4) Case reports, case series, and review papers. If a subset of patients in a study met our inclusion criteria and data from this specific population were missing in the original publication, at first, we tried to contact the corresponding author to get the necessary data. Afterward, if the relevant data was not accessible, we only included the studies when more than 50% of the participants met the inclusion criteria. However, we performed sensitivity analysis and excluded studies in which < 100% of patients met the inclusion criteria.

Data abstraction and validity assessment

Two un-blinded reviewers performed the data abstraction independently in structured

Ref.	Selection				Comparability		Outcome			Total
	1	2	3	4	5	6	7	8	9	
Afsin *et al*[12], Turkey	★	★	★		★	★	★	★	★	8
Bakhshaliyev *et al*[13], Turkey	★	★	★	★	★	★	★	★		8
Bernardini *et al*[14], Italy	★	★	★	★	★	★	★	★		8
Bun *et al*[15], France	★	★	★	★	★	★	★	★	★	9
Chorin *et al*[16], United States	★	★	★		★	★	★	★	★	8
Cipriani *et al*[17], Italy	★	★	★	★	★	★	★	★		8
Hsia *et al*[18], United States	★	★	★	★		★	★	★		7
Maraj *et al*[19], United States	★	★	★		★	★	★	★	★	8
Mercuro *et al*[20], United States	★	★	★	★	★	★	★	★		8
Ramireddy *et al*[21], United States	★	★	★	★	★	★	★	★		8
García-Rodríguez *et al*[22], Spain	★	★	★	★	★	★	★	★	★	9
Saleh *et al*[23], United States	★	★	★		★	★	★	★	★	8
Saleh *et al*[24], United States	★	★	★	★	★	★	★	★		8
Sinkeler *et al*[25], Netherlands	★	★	★	★		★	★	★		7
van den Broek *et al*[26], Netherlands	★	★	★	★	★	★	★	★		8

Figure 2 The quality assessment of the studies according to the nine-star Newcastle-Ottawa Scale scoring system.

collection forms with no divergences in the data collection method. We resolved disagreements by consensus or by involving a third person. One of the authors transferred data into the Review Manager file. We double-checked for correct data entry, comparing the data presented in the systematic review with the data extraction form. The second author spotted-check study characteristics for accuracy against the trial report. The details will be assessed by systematically reviewing the manuscripts are as follows:

The study quality was evaluated based on the following criteria: (1) The systematic review and meta-analysis based on the questions primarily described and formulated; (2) Inclusion and exclusion criteria predefined in the studies as eligibility criteria; (3) Searching the literature performed on a systematic and comprehensive approach; (4) To minimize the bias, two authors reviewed the full texts of the articles; (5) The quality of included studies were rated independently by the reviewers for appraising internal validity; (6) The characteristics and findings of the studies were listed comprehensively; (7) The publication and risk of bias were listed; and (8) Heterogeneity was also assessed. The endpoints were to determine the overall prevalence of QT prolongation and estimate the occurrence of fatal arrhythmia. Along with pooled relative risk for QT prolongation, the year of publishing, the number of patients included, and

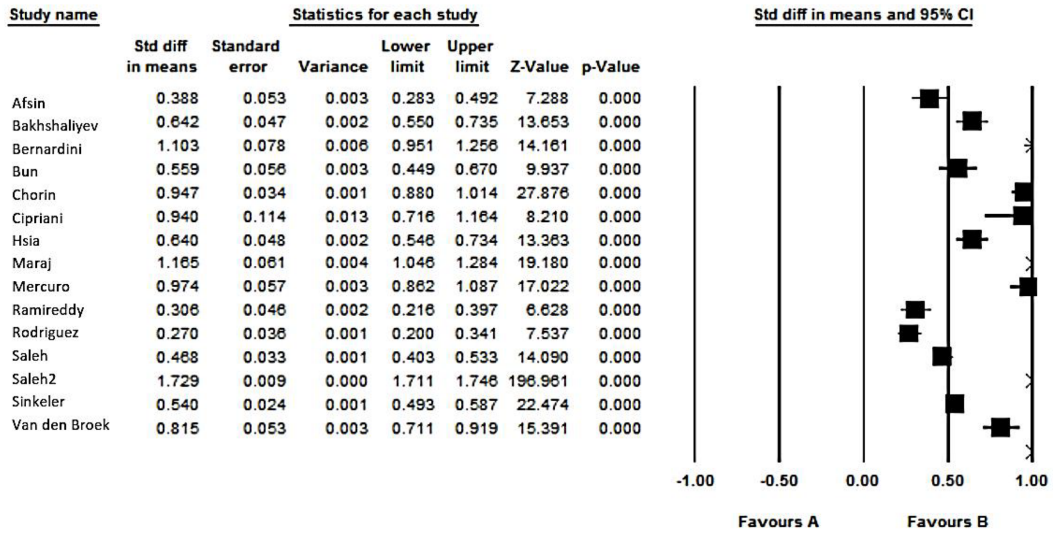


Figure 3 The pooled analysis on the difference in mean corrected QT after treatment compared to the baseline. CI: Confidence interval.

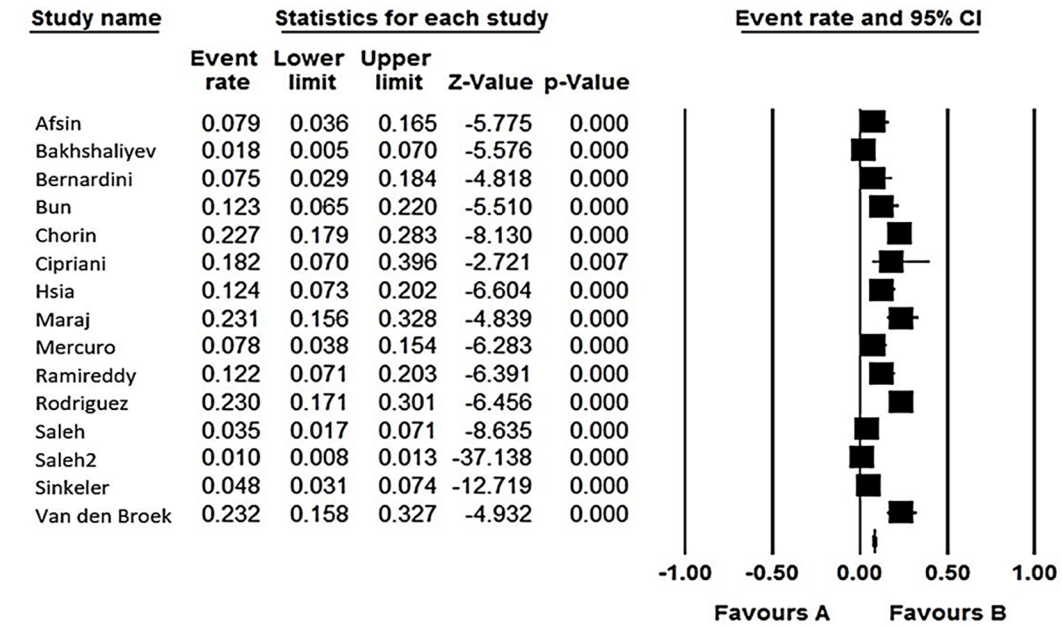


Figure 4 The pooled prevalence of corrected QT prolongation after treatment. CI: Confidence interval.

the method of the design was also pointed.

Statistical analysis

We assessed the risk of bias for each study with the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions. Any disagreement was resolved by discussion in the whole study team. We assessed the risk of bias according to the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting. We judged each potential source of bias as high, low, or unclear and provided a quote from the study report together with a justification for our judgment in the "Risk of bias" table. We summarized the risk of bias judgments across different studies for each of the domains listed. When considering therapeutical effects, we took the risk of bias into account for studies that contribute to that outcome. We contacted investigators or study sponsors to verify key study characteristics and obtain missing numerical outcome data where possible, for example, when a study is identified as abstract only. We used the RevMan calculator (version 5.3) to calculate missing standard deviations from other statistics, such as confidence intervals (CI) or *P*

-values. Where it was not possible, we imputed the missing standard deviations and explored the impact of including such studies in the overall assessment of results by a sensitivity analysis. Dichotomous variables were reported as proportions and percentages, and continuous variables as mean values. Binary outcomes from individual studies were combined with both the Mantel-Hansel fixed-effect model. The risk ratios (RRs) and 95%CI for RR were used as summary statistics to compare dichotomous variables and to determine the likelihood of each adverse event after interventions.

We used Cochran's Q test to estimate the statistical heterogeneity, complemented with the I^2 statistic. It quantifies the proportion of total variation across studies that is due to heterogeneity rather than chance. A value of I^2 of 0%–25% indicates insignificant heterogeneity, 26%–50% low heterogeneity, 51%–75% moderate heterogeneity, and 76%–100% high heterogeneity. Publication bias was assessed by the rank correlation test and also confirmed by the funnel plot analysis. The nine-star Newcastle-Ottawa Scale scoring system was employed to assess the methodological quality of all eligible studies. In this quality assessment technique, each study assessed qualitatively for the three criteria of (1) The selection of the study groups; (2) The comparability of study groups; and (3) The ascertainment of the outcome and is finally scored that the studies awarding 7 stars or over were deemed as high quality. Reported values were two-tailed, and hypothesis testing results were considered statistically significant at $P = 0.05$. Statistical analysis was performed using the Comprehensive Meta-Analysis Software (CMA, version 3.0).

RESULTS

Figure 1 demonstrates the flow diagram of the study selection. Initially, 133 articles were collected by database searching and other sources. After removing duplications, 116 records were primarily under-screened. Based on the titles and abstracts, 98 records were excluded, and the remaining 18 citations were assessed for further eligibility. Of those, 3 were also excluded due to incompleteness of the data and contents. Finally, 15 articles [12-26] were eligible for the final analysis (Figure 1). The studies included were assessed qualitatively by the QUADAS-2 tool. According to our risk of bias assessment, all 15 studies yielded good quality, and none of them had a high risk of bias. Therefore, the pooled results should be persuasive (Figure 2). In total, 15 studies (twelve retrospective and three prospective) were included in our final analysis. The anti-COVID-19 medications focused in the studies were mostly a combination of hydroxychloroquine plus azithromycin[13-24], hydroxychloroquine plus moxifloxacin[12], and hydroxychloroquine alone[25,26]. Overall, 8298 patients suffering from COVID-19 were treated with these regimens. The details of the participants are summarized in Table 1. As shown in Table 2, all studies found a significant increase in the mean QTc compared to the baseline. The weighted standard differences in means are 0.766 (95%CI: 0.394 to 1.137, $P < 0.001$), with significant heterogeneity across the studies relevant to the I^2 value of 99.33% ($P < 0.001$) (Figures 3 and 4). In this regard, the pooled prevalence rate of QT prolongation was estimated to be 9.2% (95%CI: 4.5% to 18.1%) with a significant level of heterogeneity across the studies ($I^2 = 98.10\%$, $P < 0.001$). The Egger test also detected significant publication bias for all assessments.

Across all studies, 132 patients stopped taking the medications due to QTc ≥ 500 ms or an increase of more than 60 ms in QTc. The pooled prevalence was 0.9% (95%CI: 0.6% to 1.1%) with significant level of heterogeneity across the studies ($I^2 = 81.50\%$, $P < 0.001$).

Studies did not report any mortality caused by sudden cardiac death or arrhythmogenic death. However, 4 cases of TdP and 34 cases of ventricular tachycardia/fibrillation were reported with a pooled prevalence of 0.1% < and 0.4% (95%CI: 0.2% to 0.5%), respectively.

DISCUSSION

There are controversies about the effectiveness and safety of medications used to treat COVID-19. In some cases, serious side effects following the use of these drugs may contribute to the morbidity caused by the disease and even may lead to its progression. QTc prolongation is a potential side effect of hydroxychloroquine and, also, one of the most critical complications, leading to some fatal arrhythmias like TdP.

Table 1 The baseline details of studies included in meta-analysis

Ref.	Study design	Population	Mean age	Male/female	HTN	DM	Medication
Afsin *et al*[12], Turkey	Retrospective	76	61 ± 14	32/44	41	26	Hydroxychloroquine plus Moxifloxacin
Bakhshaliyev *et al*[13], Turkey	Retrospective	109	57 ± 14	48/61	49	32	Hydroxychloroquine plus azithromycin
Bernardini *et al*[14], Italy	Retrospective	53	67 ± 12	37/16	21	6	Hydroxychloroquine plus azithromycin
Bun *et al*[15], France	Prospective	73	62 ± 14	49/24	33	19	Hydroxychloroquine plus azithromycin
Chorin *et al*[16], United States	Retrospective	251	64 ± 13	75/176	54	27	Hydroxychloroquine plus azithromycin
Cipriani *et al*[17], Italy	Retrospective	22	64 ± 11	18/4	12	6	Hydroxychloroquine plus azithromycin
Hsia *et al*[18], United States	Retrospective	105	67 ± 15	58/47	51	41	Hydroxychloroquine plus azithromycin
Maraj *et al*[19], United States	Retrospective	91	62 ± 15	51/40	42	26	Hydroxychloroquine plus azithromycin
Mercuro *et al*[20], United States	Prospective	90	60 ± 16	46/44	48	26	Hydroxychloroquine plus azithromycin
Ramireddy *et al*[21], United States	Retrospective	98	62 ± 17	60/38	59	22	Hydroxychloroquine plus azithromycin
Rodrıguez *et al*[22], Spain	Retrospective	161	63 ± 14	103/42	71	25	Hydroxychloroquine plus azithromycin
Saleh *et al*[23], United States	Prospective	201	58 ± 9	115/86	84	65	Hydroxychloroquine plus azithromycin
Saleh *et al*[24], United States	Retrospective	6476	64 ± 15	3980/2496	3184	2161	Hydroxychloroquine plus azithromycin
Sinkeler *et al*[25], Netherlands	Retrospective	397	67 ± 12	262/135	---	---	Hydroxychloroquine
van den Broek *et al*[26], Netherlands	Retrospective	95	65 ± 12	63/32	---	---	Hydroxychloroquine

Interestingly, mechanisms other than drug-related toxicity have been suggested to cause QTc prolongation. In this regard, we can mention cardiac ischemia due to direct viral invasion, electrolyte imbalances, activation of inflammatory cascades, and oxidative stress destroying myocardial tissue[27]. Studies have shown that myocardial ischemia may result in repolarization abnormality leading to cardiac arrhythmias[28]. Also, inflammatory processes, per se, may lead to disruption of cardiomyocyte ion channels by enhancing inward calcium currents and delaying outward potassium currents. It causes prolonged action potential duration and stimulation of cardiomyocytes[29]. Therefore, the occurrence of such arrhythmia may not be primarily related to drug-induced toxicity. However, our study shows a significant increase in QTc after taking hydroxychloroquine/chloroquine. These findings are in line with the previous studies, which suggest that QTc prolongation is due to drug toxicity. Further interventional studies and clinical trials are required to find an explanation for this controversy.

Besides, there is strong evidence that using azithromycin can induce QTc prolongation. According to case-control studies, azithromycin has increased the risk of QTc prolongation up to 1.5 times[30]. Of course, it seems that pre-existing cardiovascular conditions or concomitant use of other QT-prolonging drugs are the conditions for the effectiveness of this drug are in inducing arrhythmia[31].

Overall, a significant proportion of patients with COVID-19 have experienced QTc prolongation. According to our meta-analysis, 4.5% to 18.1% of COVID-19 patients have episodes of QTc prolongation, regardless of the drugs they are taking. Despite this, based on the studies, mortality from the disease does not appear to be due to arrhythmogenic events. However, providing reliable guidelines is essential for managing patients who develop QTc prolongation during treatment. A conventional cut-off for discontinuing treatment with QT-prolonging drugs is a QTc ≥ 500 ms or a rise in QTc more than 60 ms. This strategy is widely popular among the studies that we reviewed, and it led to 4 episodes of TdP and 34 episodes of ventricular tachycardia/fibrillation among 8298 participants. The authors suggest that patients with other risk factors for QTc prolongation should have continuous cardiac monitoring. Furthermore, if the patient had any indications and the clinical status deteriorated, clinicians should discontinue the medications to protect the patient from the potentially fatal arrhythmias.

Table 2 The change in QTc interval and pooled prevalence of QT prolongation following medication

Ref.	Mean QTc (base)	Mean QTc (treatment)	QT prolongation	Overall mortality	Torsades de pointes	Other ventricular arrhythmias	Sudden cardiac death	Stopped medication	Arrhythmo-genic death	Special considerations
Afsin *et al* [12], Turkey	424 ± 28	442 ± 42	6	5	0	0	0	0	0	N/A
Bakhshaliyev *et al*[13], Turkey	435 ± 32	459 ± 38	2	0	0	0	0	0	0	N/A
Bernardini *et al*[14], Italy	424 ± 24	452 ± 26	4	20	0	0	0	0	0	Atrial tachyarrhythmia: *n* = 9 (8%); Premature atrial or ventricular ectopies: *n* = 17 (15.2%); First-degree AV block: *n* = 3 (2.6%)
Bun *et al*[15], France	436 ± 44	460 ± 39	9	0	0	0	0	1	0	Counterclockwise atrial flutter: *n* = 1 (1.3%)
Chorin *et al* [16], United States	439 ± 29	473 ± 36	57	20	1	0	0	8	0	N/A
Cipriani *et al* [17], Italy	426 ± 26	450 ± 22	4	0	0	Non-sustained VT, *n* = 1	0	0	0	N/A
Hsia *et al* [18], United States	439 ± 28	459 ± 32	13	29	0	VT, *n* = 1	0	21	0	N/A
Maraj *et al* [19], United States	437 ± 25	473 ± 31	21	8	1	VF, *n* = 1	0	0	6	Bradyarrhythmia, *n* = 9 (10%)
Mercuro *et al* [20], United States	442 ± 26	473 ± 32	7	4	1	0	0	10	0	N/A
Ramireddy *et al*[21], United States	448 ± 29	459 ± 36	12	0	0	0	0	0	0	N/A
Rodrıguez *et al*[22], Spain	435 ± 25	443 ± 30	37	7	0	0	0	0	0	N/A
Saleh *et al* [23], United States	439 ± 24	463 ± 42	7	0	0	Non-sustained monomorphic VT, *n* = 1; Sustained monomorphic VT, *n* = 1	0	7	0	Atrial fibrillation: *n* = 17 (8.4%)
Saleh *et al* [24], United States	473 ± 35	532 ± 31	67	0	1	Non-sustained monomorphic VT, *n* = 18; Sustained monomorphic VT, *n* = 5; VF, *n* = 4 Sustained polymorphic VT, *n* = 1	0	58	0	N/A
Sinkeler *et al* [25], Netherlands	448 ± 34	468 ± 38	19	0	0	Non-sustained monomorphic VT, *n* = 1	0	27	0	N/A
van den Broek *et al* [26],	444 ± 32	479 ± 42	22	0	0	0	0	0	0	N/A

CONCLUSION

In conclusion, according to our systematic review and meta-analysis, a significant change in QTc interval following the use of hydroxychloroquine alone or in combination with azithromycin is highly expected that may be life-threatening. However, it should be noted that these changes may not be solely due to the toxicity of drugs. Interventional studies are required to confirm this hypothesis.

ARTICLE HIGHLIGHTS

Research background

Current evidence suggests that using hydroxychloroquine and azithromycin for coronavirus disease 2019 (COVID-19) increases the risk of cardiac arrhythmias. Previous studies reported that these drugs caused corrected QT (QTc) prolongation, leading to life-threatening conditions like torsades de pointes and sudden cardiac death. Although both *in vivo* and *in vitro* studies recommended the combination therapy of azithromycin and hydroxychloroquine, even as the first-line approach in preventing disease, it has also led to QTc prolongation.

Research motivation

In addition to cardiac monitoring, identifying patients, who are prone to side effects, helps to minimize the potential harms. By identifying susceptible individuals, it may be possible to use other drug protocols to maintain patient survival.

Research objectives

We summarize the findings about the prevalence and the risk of QTc prolongation in patients treated with hydroxychloroquine ± azithromycin. Also, we discuss the life-threatening conditions in patients taking these medications.

Research methods

We comprehensively searched Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials databases until October 31, 2020 for all eligible studies under the considered keywords COVID-19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The study protocols were established in compliance with PRISMA-P guidelines. Outcome measures were QTc prolongation, cardiac arrhythmias, or sudden cardiac death.

Research results

Fifteen studies enrolling 8298 patients with targeted COVID-19 therapeutic regimes were included. The eligible studies found a significant increase in the mean QTc interval following treatment with the described medications compared to baseline QTc with weighted standard differences in means of 0.766. The pooled prevalence rate of QTc prolongation was estimated to be 9.2% (95%CI: 4.5% to 18.1%).

Research conclusions

Hydroxychloroquine ± azithromycin regimen can significantly increase the risk of developing QTc prolongation.

Research perspectives

According to our systematic review and meta-analysis, a significant change in QTc interval following the use of hydroxychloroquine alone or in combination with azithromycin is highly expected that may be life-threatening. However, it should be noted that these changes may not be solely due to the toxicity of drugs. Interventional studies are required to confirm this hypothesis.

ACKNOWLEDGEMENTS

We are indebted to Research Development Center of Sina Hospital for their technical help. We also thank Sherry Hughes Garne for editing this paper for proper English language, grammar, punctuation, spelling, and overall style. The authors are grateful to Mrs. Mahin Ahmadi Pishkuhi for statistical consultation and for evaluating the statistical methods and tests mentioned.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 568-584

DOI: 10.13105/wjma.v9.i6.568

ISSN 2308-3840 (online)

META-ANALYSIS

MicroRNAs as prognostic biomarkers for survival outcome in osteosarcoma: A meta-analysis

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Conflict-of-interest statement: The authors deny any conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: Spain

Specialty type: Orthopedics

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

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Abstract

BACKGROUND

Osteosarcoma was considered to be one of the most prevalent malignant bone tumors in adolescents.

AIM

To explore the prognostic significance of microRNA (miRNA) in osteosarcoma.

METHODS

The literature was selected by searching online in PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, and Wanfang Database until July 1, 2021. The pooled hazard ratio (HR) with corresponding 95% confidence interval (CI) for the outcomes of overall survival (OS), disease-free survival (DFS), progression-free survival (PFS) and recurrence-free survival were calculated. Subgroup analyses were carried out to identify potential sources of heterogeneity. Publication bias was assessed by Egger's bias indicator test.

RESULTS

A total of 60 studies from 54 articles with 5824 osteosarcoma patients were included for this meta-analysis. The pooled HR for OS, DFS, PFS were 2.92 (95%CI: 2.43-3.41, P = 0.000), 3.70 (95%CI: 2.80-4.61, P = 0.000), and 3.57 (95%CI: 1.60-5.54, P = 0.000), respectively. The high miR-21 expression levels were related to poor OS in osteosarcoma (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001). Subgroup analysis demonstrated that a high expression level of miRNA correlated with worse OS (HR: 3.56, 95%CI: 2.59-4.54, P = 0.000). In addition, miRNA from tissue (HR: 3.20, 95%CI: 2.16-4.23, P = 0.000) may be a stronger prognostic biomarker in comparison with that from serum and plasma.

CONCLUSION

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Received: August 7, 2021
Peer-review started: August 7, 2021
First decision: October 2, 2021
Revised: October 8, 2021
Accepted: December 24, 2021
Article in press: December 24, 2021
Published online: December 28, 2021

P-Reviewer: Keikha M, Yu F
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

miRNA (especially miR-21) could be served as a potential prognostic biomarker for osteosarcoma. A high expression level of miRNA in tumor tissue correlated with worse OS of osteosarcoma.

Key Words: Osteosarcoma; MicroRNA; Prognosis; Biomarker; Meta-analysis

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Core Tip: Osteosarcoma is a common primary malignant bone tumor with a high degree of malignancy and poor prognosis. MicroRNA can be used as a valuable biomarker for the prognosis of osteosarcoma.

Citation: Gao SS, Zhang GX, Zhang WT. MicroRNAs as prognostic biomarkers for survival outcome in osteosarcoma: A meta-analysis. *World J Meta-Anal* 2021; 9(6): 568-584
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/568.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.568

INTRODUCTION

Osteosarcoma was considered to be one of the most prevalent malignant bone tumors in adolescents. These kind of tumors have an insidious onset, rapid progress, and a high degree of malignancy[1]. The global incidence of osteosarcoma is 4.4 cases per million people[2]. The most common locations of this tumor were the distal femur and proximal tibia, they may also involve the shoulders, mandible, skull and pelvis[3]. The main treatment therapies currently for patients with osteosarcoma are extensive tumor resection, neoadjuvant chemotherapy, and radiotherapy. However, it is well known that patients with osteosarcoma are prone to local relapse and distant metastasis (the most common being lung metastasis), and so the prognosis of osteosarcoma is still unfavorable[4]. The 5-year survival rate of patients with osteosarcoma without metastatic disease is 70%, but for patients with metastasis or relapse it is only 27.4% [5]. At present it is difficult to determine the early stage of treatable osteosarcoma, as a result, it is urgent to determine potential biomarkers that can be used to accurately assess the prognosis of osteosarcoma.

MicroRNA (miRNA) is a group of small non-coding single-stranded RNA that can interfere with the translation of many proteins after gene transcription[6]. MiRNAs participates in diverse biological processes[7], and can act as tumor suppressor genes as well as oncogenes[8]. Therefore, miRNAs may be capable of offering a sensitive method for the diagnosis, monitoring and prognosis of osteosarcoma. Previous studies have shown that miRNAs have been proposed as a valuable biomarkers for the diagnosis[9] and prognosis of osteosarcoma[10]. Although numerous studies have reported the correlation between the prognosis of osteosarcoma and miRNA expression, the results were inconsistent[11-14].

MATERIALS AND METHODS

Literature search strategy

This meta-analysis was performed according to the guidelines of the Meta-analysis of Observational Studies in Epidemiology[15]. To identify relevant studies, two investigators independently and systematically searched online in PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, and Wanfang Database until July 1, 2021. The combination of keywords used were ("osteosarcoma" or "osteosarcoma tumor") and ("microRNA" or "miRNA" or "miR") and ("prognosis" or "prognostic" or "survival" or "outcome"). Although this online search had no language restrictions, it was limited to publications with human subjects.

Inclusion and exclusion criteria

Included studies met the following criteria: (1) Studies that investigated the prognostic

value of miRNAs in osteosarcoma; (2) Assayed type either blood or tissue samples; (3) Studies that presented sufficient data to allow calculation of hazard ratio (HR) and 95% confidence interval (CI); and (4) Studies that clearly defined the cut-off and described the miRNA measurement method. Studies were excluded if they had one or more of the following criteria: (1) Duplicated or retracted studies; (2) Case reports, comments, letters, review articles or meta-analysis; (3) The obtained miRNAs were from animals or cell lines; and (4) Studies that did not have survival outcomes report or where the data presented was not sufficient to estimate HR.

Data extraction and quality assessment

All data were extracted independently by two investigators. The extracted data were as follows: first author, publication year, country, miRNA type, miRNA expression with poor prognosis (high or low), sample size, specimen type (serum, plasma, or tissue), cut off value, follow-up time (basic unit: month), clinical outcomes, detection method, survival analysis methods, and HR values of miRNAs for predicting overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), and recurrence-free survival (RFS), as well as their 95%CI and *P* values. For studies that did not directly report HR values and 95%CI, we calculated those from the given Kaplan-Meier (K-M) curves by using Engauge Digitizer[16,17]. In case of different publications that investigated the same cohort patients, we selected the most complete research. Also, when univariate and multivariate analysis were carried out at the same time, we chose the last as the more precise result. The quality of all the selected studies was assessed independently by two investigators according to the guidelines of the Newcastle-Ottawa scale (NOS)[18]. The total score of NOS ranged from 0 to 9, studies with a score ≥ 6 were considered as high quality.

Statistical analysis

All statistical analyses were carried out using STATA package version 13.0. The pooled HR with 95%CI was calculated to evaluate the correlation between miRNA expression and the clinical outcome (OS, DFS, PFS, and RFS) of osteosarcoma patients. A pooled HR < 1 implied a more favorable prognosis in patients with aberrant miRNA, and a pooled HR > 1 implied a poor prognosis. Heterogeneity among the studies was assessed using Cochran's *Q* test and Higgins's I^2 statistics. A random effect model was applied if heterogeneity was observed ($P < 0.10$ or $I^2 > 50\%$), while a fixed effect model was applied in the absence of heterogeneity ($P \geq 0.10$ or $I^2 \leq 50\%$). Subsequently, subgroup analyses for OS were carried out to identify potential sources of heterogeneity and to assess the prognostic value of different subgroups. In addition, sensitivity analysis was carried out to explore the impact of one single study on the pooled HR. Publication bias was evaluated using the Egger's bias plot[19], where $P < 0.05$ indicates significant publication bias.

RESULTS

Literature search results

The detailed literature search results are presented in Figure 1. The primary search identified 6327 articles. After excluding 2486 duplicate studies, 3841 articles remained. Secondly, 3698 studies were excluded after screening titles and abstracts, among which 2674 irrelevant research, 736 not human research, and 288 case report, letters or reviews. Subsequently, through careful reading and screening of the full text of 143 studies, 89 studies were excluded due to insufficient data. Finally, 60 studies from 54 articles were included in our meta-analysis.

Characteristics of studies

In Table 1 and Table 2, we summarized the basic information of included studies. A total of 5824 osteosarcoma patients across the 60 studies were included in this meta-analysis, among them 58 studies were conducted in China, 1 in United States and 1 in Japan. These studies have reported 51 miRNAs with different prognostic values. Among the articles included in this meta-analysis, 27 studies showed high miRNA expression and 33 studies showed low miRNA expression, indicating poor prognosis of osteosarcoma. The expression levels of miRNAs from 28 serum specimens, 6 plasmas specimens, and 26 tissue specimens were analyzed using PCR. In most of the studies, the mean/median were considered as the critical cut-off values. The follow-up time of all the studies ranged from 30 mo to 150 mo, of which 40 studies had a follow-

Table 1 Characteristics of eligible studies in this meta-analysis

Ref.	Country	MicroRNA	MicroRNA expression[1]	Sample size			Specimen	Cut off value	Follow-up (mo)	Outcome	Detection method	Survival analysis	Source of HR	NOS score
				Total	Low	High								
Yuan *et al*[20], 2012	China	miR-21	High	65	NR	NR	Serum	Median	46	OS	RT-PCR	U/M	Reported	6
Ji *et al*[21], 2013	China	miR-133a	Low	92	46	46	Tissue	Median	60	OS	RT-PCR	U	K-M curve	6
Cai *et al*[22], 2013	China	miR-210	High	92	44	48	Tissue	Median	100	OS/PFS	RT-PCR	U	K-M curve	6
Yang *et al*[23], 2013	China	miR-132	Low	166	90	76	Tissue	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	6
Li *et al*[24], 2014	China	miR-17-92	High	117	45	72	Tissue	Median	60	OS/RFS	RT-PCR	U/M	Reported	6
Xu *et al*[25], 2014	China	miR-9	High	79	39	40	Tissue	Median	60	OS	RT-PCR	M	Reported	6
Zhang *et al*[26], 2014	China	miR-133b/ 206	Low	100	NR	NR	Serum	2.7/2.8	40	OS/DFS	RT-PCR	M	K-M curve	8
Cai *et al*[27], 2014	China	miR-340	Low	92	NR	NR	Tissue	Median	100	OS/PFS	RT-PCR	M	K-M curve	7
Fei *et al*[28], 2014	China	miR-9	High	118	NR	NR	Serum	Median	80	OS	RT-PCR	U	K-M curve	6
Zhang *et al*[13], 2014	China	miR-196a/b	High	100	NR	NR	Serum	4.9/5.5	40	OS/DFS	RT-PCR	M	K-M curve	8
Ma *et al*[29], 2014	China	miR-148a	High	148	NR	NR	Plasma	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	7
Lin *et al*[30], 2014	China	miR-17-92	High	63	25	38	Tissue	Median	60	OS	RT-PCR	U	K-M curve	6
Song *et al*[31], 2014	China	miR-26a	Low	144	63	81	Tissue	Median	150	OS/DFS	RT-PCR	M	K-M curve	7
Wang *et al*[32], 2014	China	miR-214	High	82	33	49	Tissue	Median	60	OS/FFS	RT-PCR	U	K-M curve	7
Cai *et al*[33], 2015	China	miR-195	Low	166	88	78	Serum	1.44	100	OS/DFS	RT-PCR	M	K-M curve	8
Liu *et al*[34], 2015	China	miR-126	Low	122	60	62	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Sun *et al*[35], 2015	China	miR-217	Low	168	84	84	Tissue	Median	80	OS	RT-PCR	U/M	Reported	6
Wang, *et al*[36], 2015	China	miR-152	Low	80	42	38	Serum	Median	60	OS	RT-PCR	M	Reported	6
Yang *et al*[37], 2015	China	miR-221	High	108	55	53	Serum	Median	60	OS/RFS	RT-PCR	M	Reported	7
Tang *et al*[38], 2015	China	miR-27a	High	166	80	86	Serum	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	8
Wang *et al*[39], 2015	China	miR-191	High	100	42	58	Serum	3.56	60	OS/DFS	RT-PCR	M	K-M curve	8
Cao *et al*[40], 2016	China	miR-326	Low	60	25	35	Serum	Median	60	OS	RT-PCR	M	Reported	6
Dong *et al*[41], 2016	China	miR-223	Low	112	53	59	Serum	Median	60	OS	RT-PCR	M	Reported	6
Liu *et al*[42], 2016	China	miR-300	High	114	46	68	Serum	Median	60	OS/DFS	RT-PCR	U/M	Reported	6
Luo *et al*[14], 2016	China	miR-125b	Low	56	NR	NR	Plasma	Median	30	OS	RT-PCR	U/M	Reported	7

Niu *et al*[43], 2016	China	miR-95-3p	Low	133	97	36	Serum	Median	60	OS	RT-PCR	M	Reported	7
Pang *et al*[44], 2016	China	miR-497	Low	185	91	94	Serum	Median	70	OS	RT-PCR	U/M	Reported	7
Li *et al*[45], 2016	China	miR-145	low	39	19	20	Tissue	Median	60	OS	RT-PCR	U	K-M curve	7
Zhang and Wang [46], 2016	China	miR-222	High	57	28	29	Serum	Median	80	OS/DFS	RT-PCR	U/M	K-M curve	7
Ren *et al*[47], 2016	China	miR-21	High	84	33	51	Tissue	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	6
Wang *et al*[48], 2017	China	miR-34a	low	120	NR	NR	Serum	4.16	60	OS	RT-PCR	M	Reported	7
Yang *et al*[49], 2017	China	miR-203	Low	103	52	51	Tissue	Median	60	OS/DFS	RT-PCR	U/M	Reported	7
Zhu *et al*[11], 2017	China	miR-17-5p	High	62	NR	NR	Plasma	Median	70	OS/PFS	RT-PCR	U/M	Reported	6
Nakka *et al*[50], 2017	United States	miR-221	Low	32	NR	NR	Plasma	Median	150	OS	RT-PCR	M	K-M curve	6
Zou *et al*[51], 2017	China	miR-19a	High	166	80	86	Tissue	Median	100	OS/DFS	RT-PCR	U/M	Reported	7
Hu *et al*[52], 2017	China	miR-19a	High	32	NR	NR	Tissue	Median	50	OS	RT-PCR	U/M	Reported	6
Wang *et al*[53], 2017	China	miR-491	Low	102	58	44	Serum	Median	60	OS	RT-PCR	U/M	Reported	7
Wang *et al*[54], 2017	China	miR-491-5p	Low	72	36	36	Tissue/ Serum	Median	60	OS/DFS	RT-PCR	M	Reported	6
Tang *et al*[55], 2017	China	miR-490-3p	Low	148	76	72	Tissue	Median	70	OS	RT-PCR	M	K-M curve	6
Gao *et al*[56], 2018	China	miR-564	Low	217	107	110	Tissue	Median	60	OS/DFS	RT-PCR	M	K-M curve	7
Yao *et al*[57], 2018	China	miR-101	Low	152	82	70	Serum	Median	60	OS/RFS	RT-PCR	U/M	K-M curve	6
Cong *et al*[58], 2018	China	miR-124	Low	114	NR	NR	Serum	Median	60	OS/DFS	RT-PCR	M	Reported	6
Liu *et al*[59], 2018	China	miR-375	Low	95	47	48	Serum	Median	60	OS	RT-PCR	M	Reported	6
Zhong *et al*[60], 2018	China	miR-1270	High	143	69	74	Tissue	Median	70	OS	RT-PCR	U/M	Reported	7
Li *et al*[61], 2018	China	miR-542-3p	High	76	NR	NR	Serum	0.87	70	OS/DFS	RT-PCR	U/M	Reported	8
Zhou *et al*[62], 2018	China	miR-139-5p	Low	98	53	45	Serum	Median	60	OS	RT-PCR	U/M	Reported	7
Heishima *et al*[63], 2019	Japan	miR-214	High	106	NR	NR	Plasma	Median	72	OS/DFS	RT-PCR	U/M	Reported	7
Liang *et al*[12], 2019	China	miR-765	Low	33	NR	NR	Tissue	Median	72	OS	RT-PCR	U/M	Reported	6
Xu, *et al*[64], 2019	China	miR-106b	High	134	64	70	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Diao *et al*[65], 2020	China	miR-22	Low	120	60	60	Plasma	Median	60	OS	RT-PCR	U/M	Reported	6
Li *et al*[66], 2020	China	miR-1826	Low	122	69	53	Tissue	Median	60	OS	RT-PCR	U/M	Reported	7
Li *et al*[67], 2020	China	miR-629	High	110	45	65	Tissue	Median	60	OS	RT-PCR	M	Reported	7

Yang *et al*[68], 2020	China	miR-21/ 214	High	63	NR	NR	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Shi *et al*[69], 2020	China	miR-194	Low	124	69	55	Serum	Median	60	OS/DFS	RT-PCR	M	Reported	7

[1]MicroRNA expression with poor prognosis.

High: High expression; Low: Low expression; OS: Overall survival; PFS: Progression-free survival; DFS: Disease-free survival; RFS: Recurrence-free survival; NR: Not reported; M: Multivariate analysis; U: Univariate analysis; NOS: Newcastle-Ottawa scale; RT-PCR: Real-time reverse transcription-PCR.

up time of less than 5 years, and 20 studies had more than 5 years of follow-up. All of the articles were analyzed to evaluate the correlation between miRNAs and survival outcomes: 60 studies for OS, 25 studies for DFS, 4 studies for PFS, and 2 studies for RFS. The HRs and 95%CIs were directly reported in 35 studies, and the values of HRs and 95%CI in the remaining 25 studies were extracted by K-M curve. Furthermore, we assessed the quality for each study that was included in this meta-analysis and found that the NOS score varied from 6 to 8, which means the quality of each study was high.

Meta-analysis in clinical outcome of osteosarcoma

This meta-analysis was conducted based on the survival endpoint used by each study. There were 60 studies evaluated OS of osteosarcoma, the pooled HR for OS was 2.92 (95%CI: 2.43-3.41, P = 0.000). For the 25 studies that evaluated DFS of patients, the pooled HR was 3.70 (95%CI: 2.80-4.61, P = 0.000). For the studies that tested PFS (n = 4) and RFS (n = 2) as their outcome assessments, the pooled HR were 3.57 (95%CI: 1.60-5.54, P = 0.000) and 3.71 (95%CI: 0.09-7.51, P = 0.056), respectively. The pooled HR of OS studies as well as that of DFS and PFS (P < 0.05) were found to be statistically significant; however, the effect of RFS studies did not reach the level of statistical significance. The forest plots of OS are presented in Figure 2A, and the forest plots of DFS, PFS and RFS are presented in Figure 2B.

Prognostic value of miR-21 in osteosarcoma

In our meta-analysis, there were three studies that assessed miR-21 as a predictor of OS in osteosarcoma. We calculated the pooled HRs for these studies by using the random-effects model. The results suggested that high expression levels of miR-21 are correlated with poor OS (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001). In addition, there was only one study that accessed the correlation between miR-21 expression and DFS, which indicates that the high expression levels of miR-21 are correlated with poor DFS. Therefore, miR-21 may be used as a biomarker of poor prognosis in osteosarcoma.

Subgroup analyses

As shown in Table 3, six subgroup analyses of OS in osteosarcoma patients were done depending on miRNA expression, specimen, survival analysis, source of HR, follow-up, and country of publication. To analyze the prognostic value of miRNA expression in osteosarcoma, we assessed the subgroups for high expression and low expression

Table 2 Hazard ratios and 95% confidence intervals of survival outcome extracted from included studies

Ref.	MicroRNA	OS		PFS		DFS		RFS	*P*
		HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	
Yuan *et al*[20], 2012	miR-21	2.33 (1.11-4.89)	0.026						
Ji *et al*[21], 2013	miR-133a	1.87 (1.12–3.31)	0.015	2.6 (0.8–7.2)	0.02				
Cai *et al*[22], 2013	miR-210	3.3 (1.0–8.1)	0.01						
Yang *et al*[23], 2013	miR-132	4.9 (1.0–10.2)	0.001			4.1 (0.8–9.7)	0.006		
Li *et al*[24], 2014	miR-17-92	2.94 (1.51-5.75)	0.002					2.50 (1.77-6.92)	0.001
Xu *et al*[25], 2014	miR-9	4.77 (2.86–5.91)	0.002						
Zhang *et al*[26], 2014	miR-133b	5.36 (1.26-11.03)	0.02			5.69 (1.33-11.26)	0.02		
Zhang *et al*[26], 2014	miR-206	5.42 (1.31-11.28)	0.02			5.88 (1.56-12.08)	0.02		
Zhang *et al*[26], 2014	miR-133b/206	9.28 (2.69-20.79)	0.001			9.69 (2.80-21.82)	0.001		
Cai *et al*[27], 2014	miR-340	6.2 (1.4–13.9)	0.006	4.5 (1.0–9.2)	0.01				
Fei *et al*[28], 2014	miR-9	3.18 (1.35-4.42)	0.002						
Zhang *et al*[13], 2014	miR-196a	6.28 (1.62–13.39)	0.01			6.95 (1.63–14.61)	0.01		
Zhang *et al*[13], 2014	miR-196b	6.33 (1.61–13.48)	0.01			6.98 (1.65–14.82)	0.01		
Zhang *et al*[13], 2014	miR-196a/196b	9.89 (2.66–20.98)	0.001			10.09 (2.82–21.99)	0.001		
Ma *et al*[29], 2014	miR-148a	2.70 (1.41-5.17)	0.003			2.53 (1.28-5.01)	0.008		
Lin *et al*[30], 2014	miR-17-92	2.93 (1.25-6.88)	0.007						
Song *et al*[31], 2014	miR-26a	5.72 (1.01-10.94)	0.007			3.97 (1.19-9.87)	0.014		
Wang *et al*[32], 2014	miR-214	5.6 (1.2–12.9)	0.008	3.5 (1.0–8.2)	0.01				
Cai *et al*[33], 2015	miR-195	5.16 (1.92-11.88)	0.002			3.62 (1.83-9.09)	0.01		
Liu *et al*[34], 2015	miR-126	3.10 (1.11–9.02)	0.018						
Sun *et al*[35], 2015	miR-217	2.83 (1.13–7.05)	0.026						
Wang *et al*[36], 2015	miR-152	0.13 (0.02–0.70)	0.004						
Yang *et al*[37], 2015	miR-221	7.66 (1.83–15.92)	0. 01					6.82 (1.33–13.69)	0.01
Tang *et al*[38], 2015	miR-27a	2.17 (1.30-3.62)	0. 01			1.39 (0.46-4.22)	0. 01		
Wang, *et al*[39], 2015	miR-191	3.56 (1.62–7.88)	0.01			2.63 (1.28–6.06)	0.02		
Cao *et al*[40], 2016	miR-326	3.90 (1.13-12.53)	0.001						
Dong *et al*[41], 2016	miR-223	4.59 (1.84–11.45)	0.001						
Liu *et al*[41], 2016	miR-300	5.96 (2.35-10.70)	0.009			5.11 (2.14-9.48)	0.019		
Luo *et al*[14], 2016	miR-125b	0.49 (0.24-0.10)	0.049						
Niu *et al*[43], 2016	miR-95-3p	4.22 (2.31–8.07)	0.014						
Pang *et al*[44], 2016	miR-497	3.79 (1.99-8.57)	0.004						
Li *et al*[45], 2016	miR-145	2.07 (1.17-3.66)	0.0124						
Zhang and Wang[46], 2016	miR-222	1.19 (1.05-1.36)	0.008			1.23 (1.07-1.40)	0.003		
Ren *et al*[47], 2016	miR-21	6.3 (1.8–12.4)	0.001			5.8 (1.7–13.5)	0.001		
Wang *et al*[48], 2017	miR-34a	3.18 (2.87-9.56)	0.001						
Yang *et al*[49], 2017	miR-203	2.73 (1.69-8.91)	0.01			4.19 (2.91-10.12)	0.005		
Zhu *et al*[11], 2017	miR-17-5p	10.47 (2.29-47.87)	0.002	5.53 (2.02-15.16)	0.001				
Nakka *et al*[50], 2017	miR-221	0.73 (0.49-1.11)	0.139						
Zou *et al*[51], 2017	miR-19a	5.06 (1.12–11.62)	0.001			4.66 (0.88–10.97)	0.006		
Hu *et al*[52], 2017	miR-19a	2.62 (2.12-6.15)	0.037						

Wang *et al*[53], 2017	miR-491	2.96 (1.09-8.07)	0.03		
Wang *et al*[54], 2017	miR-491-5p	2.55 (1.28-4.64)	0.039	2.44 (1.21-4.51)	0.03
Wang *et al*[54], 2017	miR-491-5p	2.72 (1.30-4.92)	0.028	2.64 (1.27-5.02)	0.022
Tang *et al*[55], 2017	miR-490-3p	2.13 (1.33-7.76)	0.001		
Gao *et al*[56], 2018	miR-564	4.25 (1.66-8.63)	0.001	4.63 (1.88-9.38)	0.001
Yao *et al*[57], 2018	miR-101	3.82 (1.34-6.52)	0.014	4.23 (1.45-7.22)	0.008
Cong *et al*[58], 2018	miR-124	3.54 (1.38–5.64)	0.019	3.92 (1.57–6.39)	0.012
Liu *et al*[59], 2018	miR-375	3.27 (1.36-7.85)	0.008		
Zhong *et al*[60], 2018	miR-1270	4.34 (4.08–4.79)	0.018		
Li *et al*[61], 2018	miR-542-3p	5.98 (3.26–18.44)	0.01	6.89 (2.83–9.67)	0.018
Zhou *et al*[62], 2018	miR-139-5p	3.24 (1.81–4.76)	0.007		
Heishima *et al*[63], 2019	miR-214	2.95 (1.35-7.77)	0.005	3.51 (1.68-8.55)	0.0004
Liang *et al*[12], 2019	miR-765	0.39 (0.20-0.77)	0.007		
Xu, *et al*[64], 2019	miR-106b	2.77 (1.37-5.60)	0.005		
Diao *et al*[65], 2020	miR-22	3.31 (1.41-7.77)	0.006		
Li *et al*[66], 2020	miR-1826	2.45 (1.25-4.37)	0.014		
Li *et al*[67], 2020	miR-629	2.89 (1.13-7.42)	0.027		
Yang *et al*[68], 2020	miR-21	3.46 (2.17-11.52)	0.013		
Yang *et al*[68], 2020	miR-214	3.14 (2.01-10.26)	0.017		
Shi *et al*[69], 2020	miR-194	3.65 (1.58-5.89)	0.014	4.39 (1.90-6.91)	0.005

HR: Hazard ratio; CI: Confidence interval; OS: Overall survival; PFS: Progression-free survival; DFS: Disease-free survival; RFS: Recurrence-free survival.

levels of miRNA for OS. The high expression levels of miRNA showed a pooled HR of 3.56 (95%CI: 2.59-4.54, P = 0.000), while the low expression levels of miRNA showed a pooled HR of 2.23 (95%CI:1.74-2.72, P = 0.000) (Figure 3A). The results were found to be statistically significant for both high expression and low expression levels of miRNA; however, the high expression levels of miRNA were significantly correlated with poor prognosis in osteosarcoma. The pooled HR of different specimen of miRNAs are as follows: tissue, 3.20 (95%CI: 2.16-4.23, P = 0.000); serum, 3.04 (95%CI: 2.37-3.71, P = 0.000); plasma, 0.94 (95%CI: 0.36-1.51, P = 0.001) (Figure 3B). The result show that pooled HR for univariate analysis was 2.36 (95%CI: 1.67-3.04, P = 0.000) and for multivariate analysis was 3.29 (95%CI: 2.52-4.07, P = 0.000), and the pooled HR for univariate analysis and multivariate analysis was 2.73 (95%CI: 1.95-3.50, P = 0.000) (Figure 3C). As shown in Figure 3D, the pooled HR calculated directly from the original reported studies was 3.00 (95%CI: 2.21-3.79, P = 0.000), and the pooled HR calculated from K-M curve was 2.48 (95%CI: 1.92-3.04, P = 0.000). In addition, we found that miRNAs contributed to a different OS when the follow-up time for patients was different, the pooled HR for less than 5 years of follow-up time was 3.08 (95%CI: 2.44-3.71, P = 0.000), and the pooled HR for more than 5 years of follow-up time was 2.75 (95%CI: 1.88-3.63, P = 0.000) (Figure 3E). Furthermore, the pooled HR for OS was 3.05 in China (95%CI: 2.52-3.57, P = 0.000) (Figure 3F).

Sensitivity analysis and publication bias

We conducted a sensitivity analysis using pooled HR and by omitting single studies, and none of these studies exceeded the outliers, which indicates that the pooled results of this meta-analysis are credible. The publication bias of included studies was assessed using Egger's graphical tests, and the P values of Egger's tests were 0.987, which indicates that there was no publication bias in our meta-analysis (Figure 4).

Table 3 Subgroup analyses of the relationship between microRNA expression and overall survival.

Subgroup	Number of datasets	Heterogeneity		Pooled HR (95%CI)	*P* value
		I^2 (%)	*P* value		
Overall	60	89.0%	0.000	2.92 (2.43–3.41)	0.000
Expression					
High	27	91.4%	0.000	3.56 (2. 59–4.54)	0.000
Low	33	74.5%	0.000	2.23 (1. 74–2.72)	0.000
Specimen					
Tissue	26	92.1%	0.000	3.20 (2.16–4.23)	0.000
Serum	28	77.1%	0.000	3.04 (2.37–3.71)	0.000
Plasma	6	53.7%	0.055	0.94 (0.36–1.51)	0.001
Survival analysis					
U	6	0.0%	0.569	2.36 (1.67–3.04)	0.000
M	30	76.9%	0.000	3.29 (2.52–4.07)	0.000
U/M	24	93.9%	0.000	2.73 (1.95–3.50)	0.000
Source of HR					
Reported	35	92.6%	0.000	3.00 (2.21–3.79)	0.000
K-M curve	25	65.6%	0.000	2.48 (1.92–3.04)	0.000
Follow-up					
≤ 5 years	40	76.2%	0.000	3.08 (2.44–3.71)	0.000
> 5 years	20	94.8%	0.000	2.75 (1.88–3.63)	0.000
Country					
China	58	89.0%	0.000	3.05 (2.52–3.57)	0.000
Non-China	2	44.9%	0.178	1.24 (0.59–3.07)	0.183

OS: Overall survival; M: Multivariate analysis; U: Univariate analysis; HR: Hazard ratio; CI: Confidence interval.

DISCUSSION

Osteosarcoma is a malignant bone tumor occurring in teenagers. It is a mesenchymal tumor with histological features such as the presence of malignant mesenchymal cells and the production of bone stroma[70]. As one of the most destructive malignant tumors, osteosarcoma has a 5-year survival rate of less than 60% due to its high aggressiveness and the tendency to metastasize early[71]. It is well known that the key to the successful treatment of osteosarcoma is its accurate and timely diagnosis, and is also an important guarantee for improving prognosis and survival. This motivated us to explore more effective and/or more accurate biological biomarkers for clinical applications of osteosarcoma. MiRNAs have been used as biomarkers for the diagnosis, metastasis, and prognosis of many cancers. A large number of studies have reported the prognostic value of miRNA for osteosarcoma. However, the results are still controversial and no consensus has been reached. Therefore, we performed this meta-analysis to explore the prognostic function of miRNA in patients with osteosarcoma.

We collected all the literature on the prognostic value of miRNAs in osteosarcoma. First, we calculated the pooled HR according to the survival endpoint used in each study: the pooled HR for overall OS was 2.92 (95%CI: 2.43-3.41, $P = 0.000$), for DFS was 3.70 (95%CI: 2.80-4.61, $P = 0.000$), for PFS was 3.57 (95%CI: 1.60-5.54, $P = 0.000$), and for RFS was 3.71 (95%CI: 0.09-7.51, $P = 0.056$). It was found that the pooled HR were statistically related to the clinical outcome (OS, DFS, and PFS) of osteosarcoma ($P < 0.05$), but the impact of RFS did not reach a statistically significant level. We also evaluated the prognostic value of miR-21 in osteosarcoma. The result showed that high expression levels of miR-21 were associated with poor OS in osteosarcoma (HR =

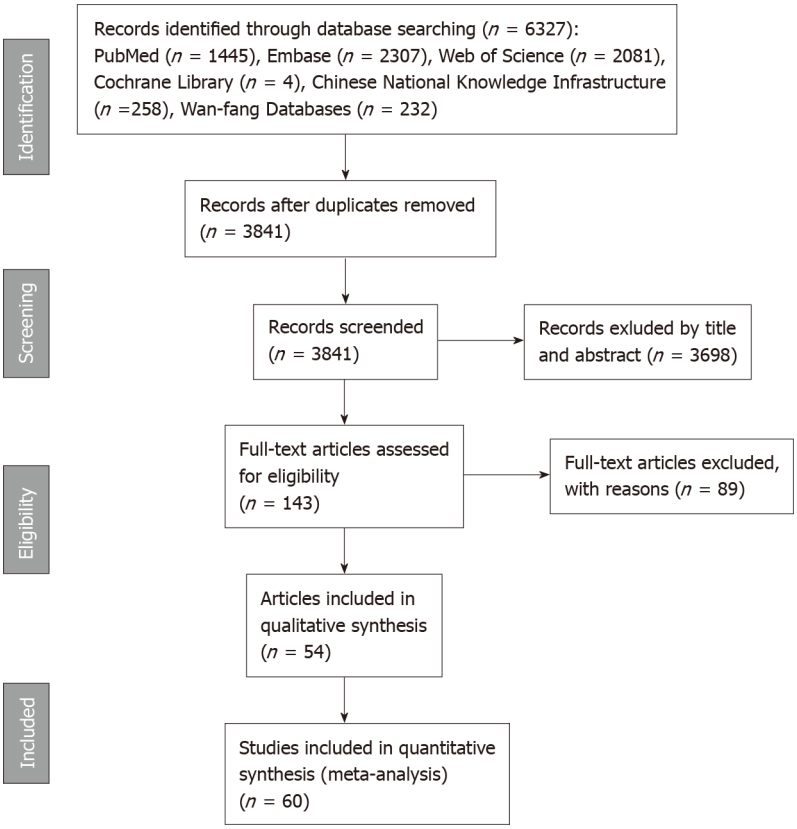


Figure 1 The flow chart of this meta-analysis to identify inclusion studies.

2.86, 95%CI: 1.20-4.53, P = 0.001), which may be used as a marker of poor prognosis in patients with osteosarcoma. MiR-21 has been demonstrated to be a tumor oncogene and plays an important role in the progression of various types of tumors[72]. A recent study suggested that, LncRNA neuroblastoma-associated transcript 1 suppresses the expression of miR-21 and also targets the miR-21-associated genes in osteosarcoma [73]. MiR-21 modulates cell invasion and migration by directly targeting reversion-inducing-cysteine-rich protein with kazal motifs and phosphatase and tensin homologue gene in osteosarcoma[74,75], it is also reduces the anti-tumor effect of cisplatin in osteosarcoma by regulating Bcl-2 expression[76].

Subsequently, due to heterogeneity of studies, we conducted a subgroup analysis of OS in patients with osteosarcoma on the basis of miRNA expression, specimens, survival rate analysis, HR source, follow-up status, and publishing country. The high expression level of miRNA shows that the combined HR is 3.56 (95%CI: 2.59-4.54, P = 0.000), while the low expression level of miRNA shows that the combined HR is 2.23 (95%CI: 1.74-2.72, P = 0.000). This indicates that the altered miRNA is related to the poor prognosis of osteosarcoma, and the high expression level of miRNA indicates a higher risk of poor prognosis of osteosarcoma. Different miRNA specimen sources may lead to different clinical outcomes. We found that miRNA from tissues (HR: 3.20, 95%CI: 2.16-4.23, P = 0.000) may be a stronger prognostic biomarker for patients with osteosarcoma. As regards the source of HR, there is no big difference between the HR directly reported in the original article and the HR extracted in the K-M survival curve, which indicates that the data extracted from the K-M survival curve according to the previous methods were reliable[17]. The length of follow-up time has different prognosis in osteosarcoma, and we found that patients with less than 5 years of follow-up have a higher risk of poor prognosis than those with more than 5 years of follow-up. This may be related to tumor aggressiveness and metastasis of osteosarcoma. The survival time of orthotopic osteosarcoma is significantly longer than that of patients with metastasis[71]. Moreover, the country of publication has no significant impact on the prognosis of patients with osteosarcoma. Finally, we conducted sensitivity analysis and publication bias and found that the combined results of the study are credible without publication bias.

This is a comprehensive meta-analysis to evaluate the prognostic function of miRNAs for osteosarcoma. Two investigators independently conducted literature screening and data extraction based on strict inclusion and exclusion criteria. We make

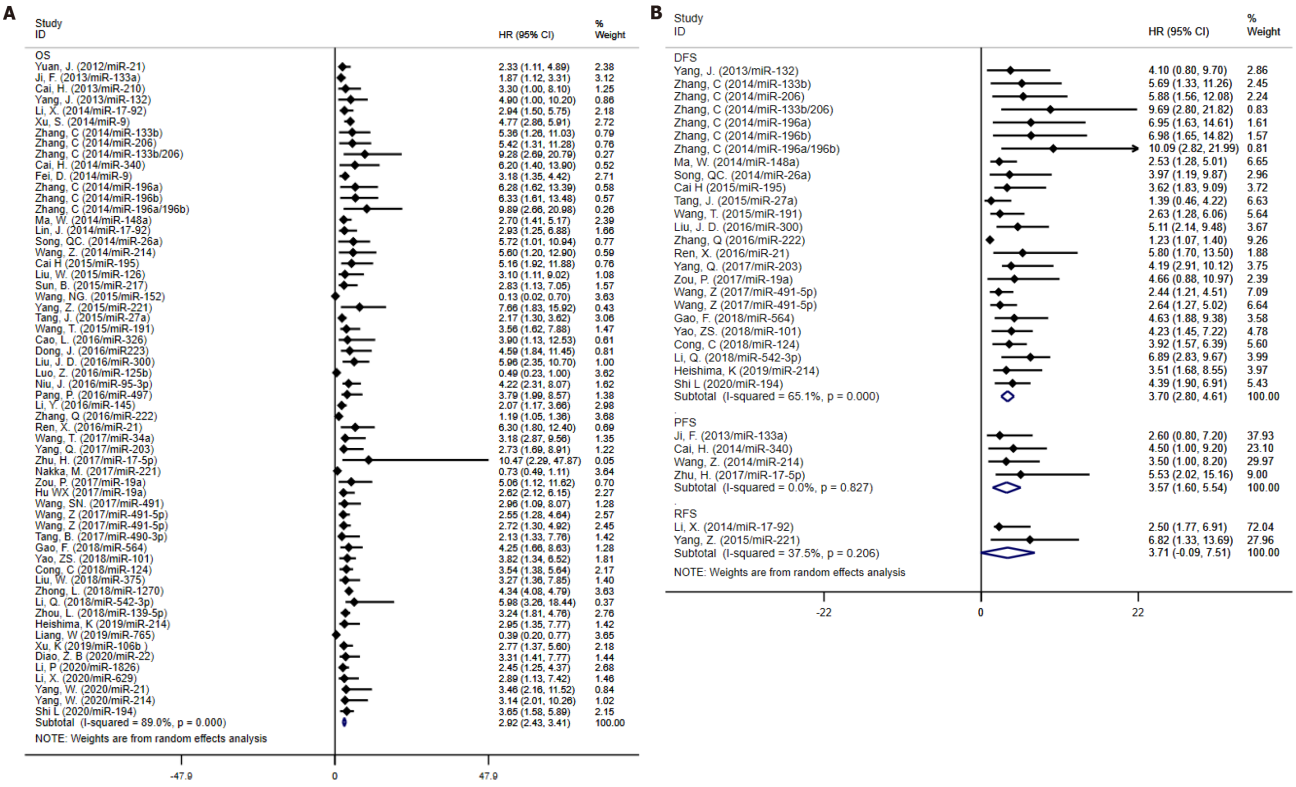


Figure 2 Forest plots for the microRNAs associated with clinical outcome. A: Forest plots for the microRNA (miRNA) associated with overall survival; B: Forest plots for the miRNAs associated with disease-free survival, progression-free survival and recurrence-free survival.

every effort to avoid publishing biases, but we acknowledge that this meta-analysis still has some limitations. First, we excluded a small number of studies that lacked original data or could not calculate HR, which may relatively reduce the convincing power of the results. Secondly, some HR data are extracted from the survival curve, which may bring small errors. Future research should be based on data obtained directly from the original article if possible. Third, although there is no obvious publication bias in this meta-analysis, positive results are more likely to be published than negative results, which may lead to an overestimation of the association between miRNAs and poor prognosis of osteosarcoma. Fourth, almost all of the 54 articles included are from China, except for one from the United States and one from Japan. Therefore, the applicability of miRNA as a prognostic indicator of osteosarcoma in other countries and regions is unclear, and our results need to be proved by more studies from other regions.

CONCLUSION

In summary, miRNA (especially miR-21), could be used as a potential prognostic biomarker for osteosarcoma. A high expression level of miRNA in tumor tissue correlated with worse OS of osteosarcoma. However, we should also focus on the low expression level of several special miRNAs. Furthermore, a large number of samples, particularly multi-center, multi-country prospective studies, will be required in the future to enhance the persuasiveness of the prognostic role of miRNA in osteosarcoma.


A
Study ID
HR (95% CI)
% Weight
High
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Zhang, Q. (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Subtotal (I-squared = 91.4%, p = 0.000) 3.56 (2.59, 4.54) 41.55
Low
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 74.5%, p = 0.000) 2.23 (1.74, 2.72) 58.45
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9
B
Study ID
HR (95% CI)
% Weight
DFS
Yang, J. (2013/miR-132) 4.10 (0.80, 9.70) 2.86
Zhang, C (2014/miR-133b) 5.69 (1.33, 11.26) 2.45
Zhang, C (2014/miR-206) 5.88 (1.56, 12.08) 2.24
Zhang, C (2014/miR-133b/206) 9.69 (2.80, 21.82) 0.83
Zhang, C (2014/miR-196a) 6.95 (1.63, 14.61) 1.61
Zhang, C (2014/miR-196b) 6.98 (1.65, 14.82) 1.57
Zhang, C (2014/miR-196a/196b) 10.09 (2.82, 21.99) 0.81
Ma, W. (2014/miR-148a) 2.53 (1.28, 5.01) 6.65
Song, QC. (2014/miR-26a) 3.97 (1.19, 9.87) 2.96
Cai H (2015/miR-195) 3.62 (1.83, 9.09) 3.72
Tang, J. (2015/miR-27a) 1.39 (0.46, 4.22) 6.63
Wang, T. (2015/miR-191) 2.63 (1.28, 6.06) 5.64
Liu, J. D. (2016/miR-300) 5.11 (2.14, 9.48) 3.67
Zhang, Q (2016/miR-222) 1.23 (1.07, 1.40) 9.26
Ren, X. (2016/miR-21) 5.80 (1.70, 13.50) 1.88
Yang, Q. (2017/miR-203) 4.19 (2.91, 10.12) 3.75
Zou, P. (2017/miR-19a) 4.66 (0.88, 10.97) 2.39
Wang, Z (2017/miR-491-5p) 2.44 (1.21, 4.51) 7.09
Wang, Z (2017/miR-491-5p) 2.64 (1.27, 5.02) 6.64
Gao, F. (2018/miR-564) 4.63 (1.88, 9.38) 3.58
Yao, ZS. (2018/miR-101) 4.23 (1.45, 7.22) 4.78
Cong, C (2018/miR-124) 3.92 (1.57, 6.39) 5.60
Li, Q. (2018/miR-542-3p) 6.89 (2.83, 9.67) 3.99
Heishima, K (2019/miR-214) 3.51 (1.68, 8.55) 3.97
Shi L (2020/miR-194) 4.39 (1.90, 6.91) 5.43
Subtotal (I-squared = 65.1%, p = 0.000) 3.70 (2.80, 4.61) 100.00
PFS
Ji, F. (2013/miR-133a) 2.60 (0.80, 7.20) 37.93
Cai, H. (2014/miR-340) 4.50 (1.00, 9.20) 23.10
Wang, Z. (2014/miR-214) 3.50 (1.00, 8.20) 29.97
Zhu, H. (2017/miR-17-5p) 5.53 (2.02, 15.16) 9.00
Subtotal (I-squared = 0.0%, p = 0.827) 3.57 (1.60, 5.54) 100.00
RFS
Li, X. (2014/miR-17-92) 2.50 (1.77, 6.91) 72.04
Yang, Z. (2015/miR-221) 6.82 (1.33, 13.69) 27.96
Subtotal (I-squared = 37.5%, p = 0.206) 3.71 (-0.09, 7.51) 100.00
NOTE: Weights are from random effects analysis
-22
0
22
C
Study ID
HR (95% CI)
% Weight
U
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Subtotal (I-squared = 0.0%, p = 0.596) 2.36 (1.67, 3.04) 12.31
M
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 76.9%, p = 0.000) 3.29 (2.52, 4.07) 41.60
U/M
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Zhang, Q (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Subtotal (I-squared = 93.9%, p = 0.000) 2.73 (1.95, 3.50) 46.10
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9
D
Study ID
HR (95% CI)
% Weight
Reported
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 92.6%, p = 0.000) 3.00 (2.21, 3.79) 62.09
K-M curve
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Zhang, Q. (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Subtotal (I-squared = 65.6%, p = 0.000) 2.48 (1.91, 3.04) 37.91
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9

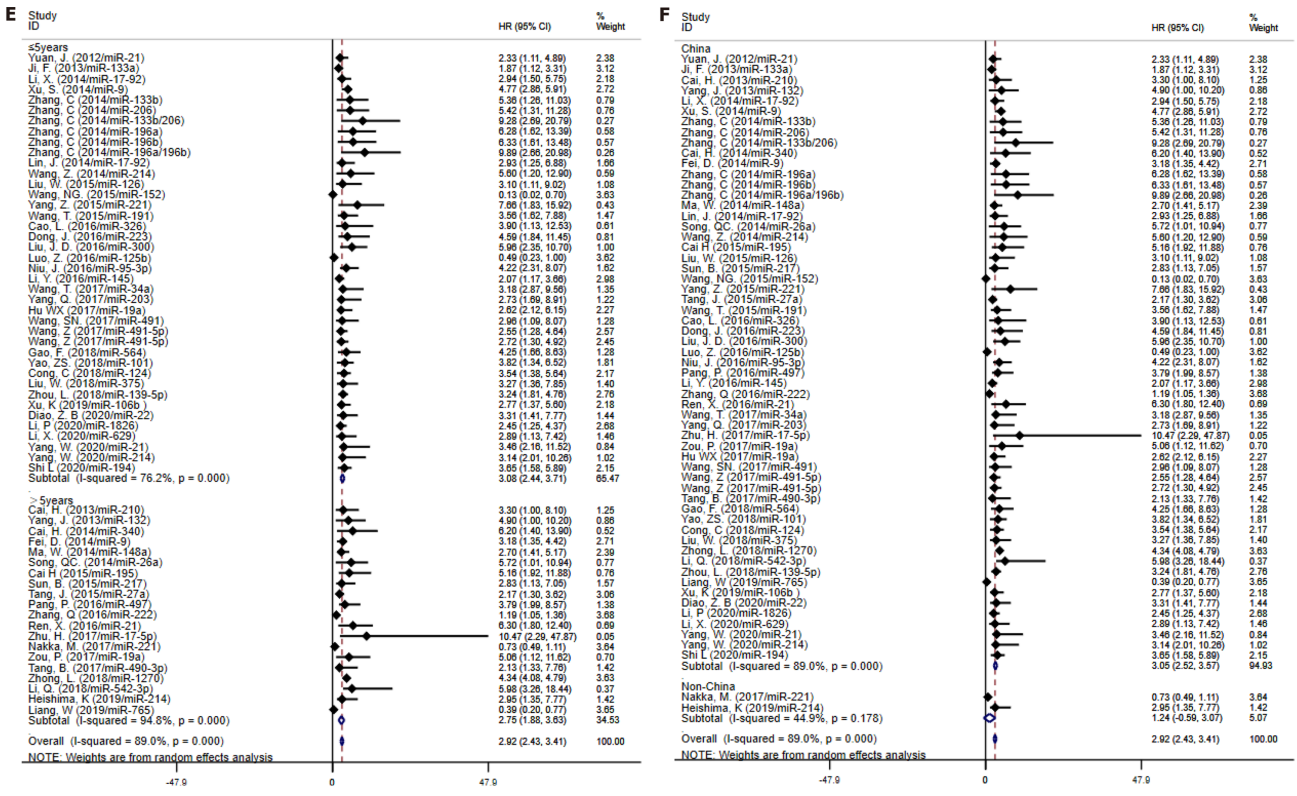


Figure 3 Forest plot of subgroup analysis. A: Subgroup analysis based on microRNA expression; B: Subgroup analysis based on specimen type; C: Subgroup analysis based on survival analysis; D: Subgroup analysis based on source of hazard ratio; E: Subgroup analysis based on follow-up; F: Subgroup analysis based on country of publication.

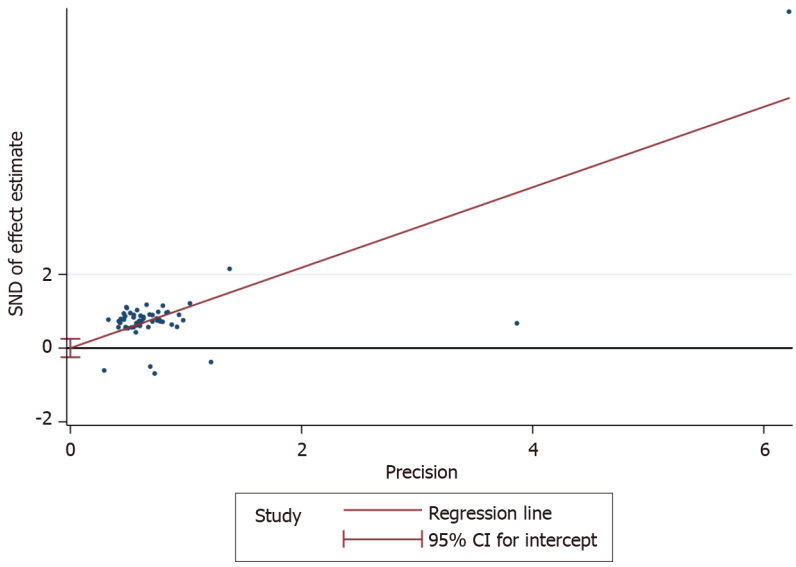


Figure 4 Egger's test of publication bias.

ARTICLE HIGHLIGHTS

Research background

Osteosarcoma is one of the most common primary malignant bone tumors in children, with rapid progress and poor prognosis. microRNA (miRNA) has been proposed as a valuable biomarker for the prognosis of osteosarcoma, but the results are not consistent.

Research motivation

We wished to gain a detailed insight into the effect of circulating miRNA on the prognosis of patients with osteosarcoma.

Research objectives

The aim of this meta-analysis was to study the prognostic value of miRNA expression in patients with osteosarcoma.

Research methods

A literature search was conducted to identify studies published through July 1, 2021. Random-effects meta-analyses were conducted for combinations of the outcomes of overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), and recurrence-free survival in osteosarcoma patients. Subgroup analyses were conducted to identify sources of heterogeneity. Publication bias was assessed by Egger's bias indicator test.

Research results

A total of 60 studies were included for this meta-analysis. The pooled HR for OS, DFS, PFS were 2.92 (95%CI: 2.43-3.41, P = 0.000), 3.70 (95%CI: 2.80-4.61, P = 0.000), and 3.57 (95%CI: 1.60-5.54, P = 0.000), respectively. The high expression levels of miR-21 were associated with poor OS in osteosarcoma (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001).

Research conclusions

Circulating miRNA could be used as a potential prognostic biomarker for osteosarcoma.

Research perspectives

Evidence from later studies could consolidate these conclusions. A large number of samples prospective studies are needed in the future to enhance the persuasiveness of our conclusion.

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World Journal of Meta-Analysis

Submit a Manuscript: https://www.f6publishing.com

World J Meta-Anal 2021 December 28; 9(6): 585-597

DOI: 10.13105/wjma.v9.i6.585

ISSN 2308-3840 (online)

META-ANALYSIS

Efficacy and safety of fingolimod in stroke: A systemic review and meta-analysis

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Conflict-of-interest statement: On behalf of all authors, the corresponding author states that there is no conflict of interest to disclose.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Supported by the National Key R&D Program of China, No. 2018YFC1312601; and the Project of Science and Technology Department of Qinghai Province, No. 2020-ZJ-774.

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Abstract

BACKGROUND

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration. However, no evidence-based efficacy and safety evaluation of fingolimod in stroke patients is currently available.

AIM

To determine whether fingolimod could promote reduction of infarction lesion or hematoma and improve neurological prognosis in stroke patients.

METHODS

Data extracted for treatment effect included count of T-lymphocytes with cluster of differentiation 8 expression ($CD8^+$ T cells, $\times 10^6$/mL), lesion volume (infarction or hematoma, mL), and modified Barthel indexes. Data extracted for safety was risk ratio (RR). Overall standard mean difference (SMD) with its 95% confidence interval (95%CI) and pooled effect with its 95%CI were calculated with a fixed-effects model. *I*-square (I^2) was used to test the heterogeneity. Funnel plot symmetry and Egger's regression were used to evaluate publication bias.

RESULTS

Four high-quality randomized controlled trials were included. There was a significant difference in $CD8^+$ T cell count ($I^2 = 0$, overall SMD = -3.59, 95%CI: -4.37-2.80, $P = 0.737$) and modified Barthel index ($I^2 = 0$, overall SMD = 2.42, 95%CI: 1.63-3.21, $P = 0.290$) between the fingolimod and control groups. However, there was no significant difference in lesion volume ($I^2 = 10.6\%$, overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$), fever (pooled RR = 0.93, 95%CI: 0.97-2.32, $P = 0.864$),

Country/Territory of origin: China

Specialty type: Clinical neurology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D, D
Grade E (Poor): 0

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Received: August 18, 2021
Peer-review started: August 18, 2021
First decision: November 2, 2021
Revised: November 13, 2021
Accepted: December 24, 2021
Article in press: December 24, 2021
Published online: December 28, 2021

P-Reviewer: Cheungpasitporn W, Liu JM
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Liu M

suspected lung infection (pooled RR = 0.90, 95%CI: 0.33-2.43, P = 0.876), or any adverse events occurring at least once (pooled RR = 0.82, 95%CI: 0.36-1.87, P = 0.995) between the fingolimod and control groups. There was no publication bias. All of the results were stable as revealed by sensitivity analysis.

CONCLUSION
Fingolimod improves neurological function in stroke patients without promotion of lesion absorption. Taking fingolimod orally (0.5 mg/d, 3 consecutive days) is safe except for patients with rare severe adverse events.

Key Words: Acute ischemic stroke; Intracerebral hemorrhage; Fingolimod; Meta-analysis; Treatment

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Core Tip: Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. Our systemic review and meta-analysis of recent randomized controlled trials found that fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively; however, we did not find the evidence that fingolimod could promote infarction lesion or hematoma absorption. In general, oral fingolimod (0.5 mg/d, 3 consecutive days) was safe in stroke patients except for some rare severe adverse events.

Citation: Zhao K, Guo Y, Yang MF, Zhang Q. Efficacy and safety of fingolimod in stroke: A systemic review and meta-analysis. *World J Meta-Anal* 2021; 9(6): 585-597
URL: https://www.wjgnet.com/2308-3840/full/v9/i6/585.htm
DOI: https://dx.doi.org/10.13105/wjma.v9.i6.585

INTRODUCTION

Stroke, which mainly consists of acute ischemic stroke (AIS) with cerebral infarction and hemorrhagic stroke with intracerebral hematoma (ICH), is a common neurological disease with a high mortality and disability rate. Especially, during the coronavirus disease 2019 pandemic, related coagulopathy might be associated with ischemic stroke, cerebral hemorrhage, and cerebral venous thrombosis, which aggravated the financial burden in the world[1,2]. For severe stroke patients whose condition has deteriorated rapidly, surgical intervention should be performed, which is considered the best way to save lives[3]. Thus, to reduce the risk of disease deterioration and promote the recovery of nervous system function, it is important to perform timely intervention at the early stage of stroke.

Fingolimod is a newly approved drug for the treatment of multiple sclerosis. It can be metabolized to the active metabolite of fingolimod phosphate *via* sphingosine kinase. Fingolimod phosphate has a high affinity for sphingosine-1-phosphate receptors 1, 3, 4, and 5[4]. Sphingosine-1-phosphate and its receptors can regulate lymphocyte migration *via* upstream cell signaling pathways. Thus, fingolimod phosphate can block the ability of lymphocytes to enter or exit the lymph nodes, which can reduce the number of lymphocytes in peripheral blood[5]. Although fingolimod can be used for the treatment of multiple sclerosis with unknown mechanism, its role as an immunomodulator might involve the reduction of the migration of lymphocytes to central nervous system[6].

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis[7]. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration[8]. However, evidence-based efficacy and safety evaluation of fingolimod in stroke patients is currently not available. We hypothesized that fingolimod could effectively and safely promote reduction of infarction lesion or hematoma and improve neurological prognosis in AIS or ICH patients by reducing

lymphocyte infiltration. In this study, we performed a systemic review and meta-analysis of recent randomized controlled trials (RCTs) to confirm this hypothesis.

MATERIALS AND METHODS

Protocol used

This systemic review and meta-analysis were performed referring to the protocol published in the database of International Platform of Registered Systematic Review and Meta-analysis Protocols (INPLASY, https://inplasy.com/).

Literature search

A literature search was performed in the three public electronic databases: PubMed, Embase, and Cochrane. The strategy of literature search in PubMed was as follows: ("Fingolimod Hydrochloride"[Mesh]) OR (((((2-Amino-2-(2-(4-octylphenyl)ethyl)-1,3-propanediol hydrochloride[Title/Abstract]) OR (FTY?720[Title/Abstract])) OR (Gilenya[Title/Abstract])) OR (Gilenia[Title/Abstract])) OR (Fingolimod [Title/Abstract])) AND (("Stroke"[Mesh]) OR ((((((((((((((((((Stroke*[Title/Abstract]) OR (Cerebrovascular Accident*[Title/Abstract])) OR (CVA* [Title/Abstract])) OR (Cerebrovascular Apoplexy[Title/Abstract])) OR (Apoplexy, Cerebrovascular [Title/Abstract])) OR (Vascular Accident, Brain[Title/Abstract])) OR (Brain Vascular Accident*[Title/Abstract])) OR (Vascular Accidents, Brain[Title/Abstract])) OR (Cerebrovascular Stroke*[Title/Abstract])) OR (Stroke*, Cerebrovascular[Title/Abstract])) OR (Apoplexy[Title/Abstract])) OR (Cerebral Stroke*[Title/Abstract])) OR (Stroke*, Cerebral[Title/Abstract])) OR (Stroke*, Acute[Title/Abstract])) OR (Acute Stroke*[Title/Abstract])) OR (Cerebrovascular Accident*, Acute[Title/Abstract])) OR (Acute Cerebrovascular Accident*[Title/Abstract]))). The strategy of literature search in Embase was as follows: (exp Stroke or Stroke$:ab,ti or 'Cerebrovascular Accident$':ab,ti or CVA$:ab,ti or 'Cerebrovascular Apoplexy':ab,ti or 'Apoplexy, Cerebrovascular':ab,ti or 'Vascular Accident, Brain':ab,ti or 'Brain Vascular Accident$':ab,ti or 'Vascular Accidents, Brain':ab,ti or 'Cerebrovascular Stroke$':ab,ti or 'Stroke$, Cerebrovascular':ab,ti or Apoplexy:ab,ti or 'Cerebral Stroke$':ab,ti or 'Stroke$, Cerebral':ab,ti or 'Stroke$, Acute':ab,ti or 'Acute Stroke$':ab,ti or 'Cerebrovascular Accident$, Acute':ab,ti or 'Acute Cerebrovascular Accident$':ab,ti) or (exp 'Cerebral Hemorrhage' or 'Hemorrhage$, Cerebrum':ab,ti or 'Cerebrum Hemorrhage$':ab,ti or 'Cerebral Parenchymal Hemorrhage$':ab,ti or 'Hemorrhage$, Cerebral Parenchymal':ab,ti or 'Parenchymal Hemorrhage$, Cerebral':ab,ti or 'Intracerebral Hemorrhage$':ab,ti or 'Hemorrhage$, Intracerebral':ab,ti or 'Hemorrhage$, Cerebral':ab,ti or 'Cerebral Hemorrhage$':ab,ti or 'Brain Hemorrhage$, Cerebral':ab,ti or 'Cerebral Brain Hemorrhage$':ab,ti or 'Hemorrhage$, Cerebral Brain':ab,ti).

Study selection

The articles involving the efficacy and safety of fingolimod in the treatment of stroke patients were screened. The inclusion criteria were: (1) Language and regions were not restricted; (2) Date of publication was up to December 31, 2020; (3) RCTs; (4) Patients suffered from AIS or ICH and the onset time was less than 6 h; (5) Fingolimod was used as the clinical intervention and the basic treatment methods were standard treatment according to the current American Heart Association guidelines; and (6) Outcomes involved assessment of immune cells, lesion volume, neurological function, and adverse events. The exclusion criteria were: (1) Duplicated publications; (2) Reviews, comments, letters, case reports, protocols of clinical trials, and conference papers; (3) Animal experiments; and (4) Articles with data irrelevant to this meta-analysis.

Data extraction

In consideration of the pharmacological action of fingolimod, absolute immunological cell counts ($\times 10^6$/mL) in blood might be the most intuitive index. Especially, T-lymphocytes with cluster of differentiation 8 expression ($CD8^+$ T cells) play a key role in damaging the nervous system due to its cell killing effect, which could result in inflammatory edema[9]. The edema around the infarction or hematoma could result in serious neurologic impairment in stroke patients[10,11]. Thus, a rapid reduction of absolute infarction or hematoma volume might be the key of treatment in stroke patients. Lesion volume (mL) was defined as absolute infarction volume in AIS patients or absolute hematoma in ICH patients. The National Institute of Health stroke

scale (NIHSS) is the specialized assessment to measure recovery of neurological function[12]. The Modified Barthel index (mBI) is one of the earliest and the most commonly used methods for assessing ADL (activity of daily living) in rehabilitation medicine, which has high validity and reliability in assessment of stroke[13]. Although risk ratio (RR) is often used as an effect size in cohort studies, it might be significant to assess the probability of adverse events like the number of patients to be harmed in clinical trials[14]. In this study, the data extracted included $CD8^+$ T cell count, lesion volume (infarction or hematoma), NIHSS, mBI, and RR of safety. The data at time points near baseline, which might reflect condition of stroke at early treatment, were extracted. In addition, some confounders, which might result in errors, were adjusted, including characteristics of participation, period of treatment, and other factors. If the original data could not be acquired, other data were also extracted such as the difference between data at baseline and those at other time point.

Risk of bias

The quality assessment of included articles was performed *via* the Cochrane Collaboration's Tool of Assessing Risk of Bias using Review Manager 5.3 before data extraction.

Statistical analysis

We made the definition that the counts of studies selected or those with data extracted were positive events (+). The others were negative events (-). Kappa was calculated to test the agreement between Kai Zhao and Yu Guo. Kappa > 0.6 meant a high agreement. Zhang Q would make the final decision. Continuous variables with a normal distribution are expressed as the mean ± SD. Statistical difference of data before treatment in the intervention and control groups was tested by one-way analysis of variance (ANOVA) using SigmaStat version 4.0. If there was no statistical difference, data after treatment were directly used for meta-analysis. Relative numbers and their 95% confidence intervals (95%CI) are used to describe count data. Meta-analysis was performed using corresponding modules in Software for Statistics and Data Science (Stata, version 15.1; College Station, Texas 77845, United States). The overall standard mean difference (SMD) with its 95%CI and pooled effect with its 95%CI were calculated with a fixed-effects model. *I*-square (I^2) was used to test the heterogeneity. Sensitivity analysis was performed to evaluate the stability of overall results by recalculating the overall SMD or pooled effect of the remaining studies after omitting the study with the highest quality or the fixed-effects model was switched to a random-effects model. Funnel plot symmetry and Egger's regression were used to evaluate publication bias. The methods to reduce heterogeneity and enhance sensitivity were the deletion of studies with publication bias or studies with the lowest quality and subgroup analysis. All *P* values were two-sided with a significant level at 0.05.

RESULTS

Study selection and characteristics

Totally, 185 articles were retrieved from three databases according to the strategy. After screening according to the inclusion and exclusion criteria, four articles[15-18] reporting RCTs were enrolled ultimately (Figure 1). The Kappa of agreement of studies included by Kai Zhao and Yu Guo was 0.601 ($P < 0.001$; Table 1). One study emphasized the changes of inflammatory factors in patients with ICH after taking fingolimod orally[19]. One study emphasized the role of rehabilitation nursing in AIS patients after taking fingolimod orally[20]. The assessment of article quality *via* Cochrane Collaboration's Tool of Assessing Risk of Bias is shown in Figure 2. Three studies (Ying Fu *et al*[15], Ying Fu *et al*[16], and Zilong Zhu *et al*[17]) were evaluated as having insignificant risk bias in allocation concealment except for low risk in one study (De-Cai Tian *et al*[18]). There was no bias in performance, detection, attrition, or reporting. Other risk biases were insignificant in all studies. The articles finally included were published in 2014 to 2018 (Table 2). There were 67 stroke patients, including 56 AIS patients and 11 ICH patients, whose age ranged from 49 to 74 years. The period of oral fingolimod were 3 consecutive days after hospitalization. The oral dose was 0.5 mg/d. Conclusion with the same tendency was that fingolimod could effectively and safely treat AIS or ICH.

Table 1 Agreement of studies included by Zhao K and Guo Y

Zhao K	Guo Y		Total
	+	-	
+	4	3	7
-	2	176	179
Total	6	179	185

Kappa = 0.601, $P < 0.001$.

Table 2 Characteristics of studies used to perform the meta-analysis

Ref.	Publication	Fingolimod group	Types of stroke	Age (mean ± SD)	Sex (male %)	Dose of fingolimod	Conclusion
Fu *et al*[15]	2014	11	AIS	62.3 ± 8.0	73	0.5 mg/d orally, 3 consecutive days after hospitalization	It could safely limit secondary tissue injury, decrease microvascular permeability, attenuate neurological deficits, and promote recovery.
Fu *et al*[16]	2014	11	ICH	60.7 ± 12.3	36	0.5 mg/d orally, 3 consecutive days after hospitalization	It could safely reduce PHE, attenuate neurologic deficits, and promote recovery.
Zhu *et al* [17]	2015	22	AIS	60.0 ± 2.5	59	0.5 mg/d orally, 3 consecutive days after hospitalization	In this pilot study, combination therapy of fingolimod and alteplase was well tolerated, which attenuated reperfusion injury and improved clinical outcomes in AIS patients.
Tian *et al* [18]	2018	23	AIS	67 ± 6.7	39	0.5 mg/d orally, 3 consecutive days after hospitalization	Fingolimod may enhance the efficacy of alteplase administration in the 4.5- to 6-h time window in patients with a proximal cerebral arterial occlusion and salvageable penumbral tissue by promoting both anterograde reperfusion and retrograde collateral flow.

SD: Standard deviation.

Meta-analysis

Due to that some data were expressed *via* only one figure, some original data could not be acquired directly, which may result in missing of some data. The Kappa of agreement of data extraction between Zhao K and Guo Y was 0.634 ($P < 0.001$; Table 3). To acquire a more accurate result of meta-analysis, we tested the statistical difference of $CD8^+$ T cell counts at baseline and 1 day after taking fingolimod orally, lesion volume at baseline and 7 d after taking fingolimod orally, and mBI scores at baseline and 90 d after taking fingolimod orally between studies *via* ANOVA. Unfortunately, there were significant statistical differences ($P < 0.05$) in all of continuous variables. Finally, we calculated difference between data at different time points manually to perform meta-analysis (Table 4). RRs and their 95%CIs of different adverse events, which consisted of fever, suspected lung infection, all adverse events occurring at least once, and other serious events involving hemorrhage of the digestive tract, cerebral hernia, bradycardia, atrial flutter, and thrombocytopenia, were calculated directly by referring to the count of adverse events occurring in patients of the fingolimod groups and control groups. Since no patients developed bradycardia, atrial flutter, or thrombocytopenia in the control groups, RRs of these adverse events were not calculated (Table 5).

Analysis with a fixed-effects model showed that there was no heterogeneity ($I^2 = 0$, Figure 3) in the two studies used for comparing overall SMD difference in $CD8^+$ T cell counts and lesion volume except for two articles used for comparing overall SMD difference in mBI scores ($I^2 = 10.6\%$, Figure 3). There was a significant difference in $CD8^+$ T cell counts (overall SMD = -3.59, 95%CI: -4.37--2.80, $P = 0.737$, Figure 3) and mBI (overall SMD = 2.42, 95%CI: 1.63-3.21, $P = 0.290$, Figure 3) between the fingolimod and control groups. However, there was no significant difference in lesion volume between the fingolimod and control groups (overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$, Figure 3). Pooled RRs and their 95%CIs were calculated with a fixed-effects model. The RRs of fever ($I^2 = 0$, pooled RR = 0.93, 95%CI: 0.97-2.32, $P = 0.864$, Figure 4),

Table 3 Agreement of data extraction between Zhao K and Guo Y

Zhao K	Guo Y		Total
	+	-	
+	16	5	21
-	3	20	23
Total	19	25	44

Kappa = 0.634, $P < 0.001$.

Table 4 Changes of T-lymphocytes with cluster of differentiation 8 expression/infarction or hematoma volume/modified Barthel index scores in the fingolimod and control groups

Ref.	Size of fingolimod group	Fingolimod group	Size of control group	Control group
$CD8^+$ T cell count (mean ± SD, × 10^6 /mL)[1]				
Fu *et al*[15]	11	-0.16 ± 0.04	11	0.08 ± 0.08
Tian *et al*[18]	23	-0.09 ± 0.02	23	-0.02 ± 0.02
Lesion Volume (mean ± SD, mL)[2]				
Fu *et al*[15]	11	9.91 ± 79.62	11	25.45 ± 75.45
Fu *et al*[16]	11	-1.97 ± 6.25	12	-1.35 ± 1.77
mBI score (mean ± SD)[3]				
Fu *et al*[15]	11	72.7 ± 7.5	11	45.3 ± 10.9
Fu *et al*[16]	11	75.2 ± 4.7	12	65.4 ± 4.8

[1]The outcome equaled $CD8^+$ T cell count at 1 d after taking fingolimod orally minus $CD8^+$ T cell count at baseline.
[2]Lesion volume consisted of infarct volume of AIS and hematoma volume of ICH. The outcome is lesion volume at 7 d after taking fingolimod orally minus lesion volume at baseline.
[3]The outcome is mBI score at 90 days after fingolimod taken orally minus mBI score at baseline.
$CD8^+$ T cell: T-lymphocytes with cluster of differentiation 8 expression. Lesion volume: Infarction or hematoma volume; mBI score: Modified Barthel index score.

suspected lung infection ($I^2 = 0$, pooled RR = 0.90, 95%CI: 0.33-2.43, $P = 0.876$, Figure 4), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.82, 95%CI: 0.36-1.87, $P = 0.995$, Figure 4) showed no significant difference between the fingolimod and control groups. Meta-analysis was not performed in RRs of other serious events because there was only one study reporting hemorrhage of the digestive tract or cerebral hernia and RRs of bradycardia, atrial flutter, and thrombocytopenia could not be calculated.

Publication bias and influence analysis

There was a symmetrical distribution in funnel plots of continuous variables (Figure 4A) and RRs (Figure 4B). Because only two studies were used to perform meta-analysis of treatment effect, we recalculated overall SMD of $CD8^+$ T cell count ($I^2 = 0$, overall SMD = -3.59, 95%CI: -4.37--2.80, $P = 0.737$, Table 6), lesion volume ($I^2 = 0$, overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$, Table 6), and mBI scores ($I^2 = 10.6\%$, overall SMD = 2.43, 95%CI: 1.59-3.26, $P = 0.290$, Table 6) after the fixed-effects model was switched to a random-effects model. Pooled RRs and their 95%CIs of fever ($I^2 = 0$, pooled RR = 0.93, 95%CI: 0.37-2.32, $P = 0.723$, Table 6), suspected lung infection ($I^2 = 0$, pooled RR = 0.90, 95%CI: 0.33-2.43, $P = 0.723$, Table 6), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.82, 95%CI: 0.36-1.87, $P = 0.969$, Table 6) were analyzed using the same method as above. In addition, for sensitivity analysis, the pooled effect of the remaining studies was recalculated after omitting the study with the highest quality because more than two studies were used to perform meta-analysis of treatment safety. We considered that the study might be assessed to have higher quality for its larger number of included patients in studies with the same

Table 5 Complications and adverse events in the fingolimod and control groups

Ref.	Size of fingolimod group	Events of fingolimod group	Size of control group	Events of control group	Risk ratio (95%CI)
Fever					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	3	12	5	0.64 (0.12, 3.34)
Zhu *et al*[17]	22	5	25	5	1.15 (0.29, 4.51)
Suspected lung infection					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	3	12	5	0.64 (0.12, 3.34)
Zhu *et al*[17]	22	3	25	3	1.17 (0.21, 6.39)
Adverse events occurring at least once					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	4	12	6	0.72 (0.12, 4.38)
Zhu *et al*[17]	22	6	25	8	0.84 (0.25, 2.81)
Tian *et al*[18]	23	3	23	4	0.76 (0.15, 3.81)
Other serious events					
Hemorrhage of digestive tract					
Zhu *et al*[17]	22	1	25	3	0.42 (0.04, 4.30)
Cerebral hernia					
Tian *et al*[18]	23	2	23	6	0.35 (0.06, 1.90)
Bradycardia					
Fu *et al*[16]	11	1	12	0	N/A
Atrial flutter					
Tian *et al*[18]	23	2	23	0	N/A
Thrombocytopenia					
Tian *et al*[18]	23	2	23	0	N/A

assessment in Cochrane Collaboration's Tool of Assessing Risk of Bias. Finally, after omitting the study with the highest quality, we recalculated pool RRs and their 95%CIs of fever (Zhu *et al*[17] omitted, $I^2 = 0$, pooled RR = 0.78, 95%CI: 0.23-2.68, $P = 0.723$, Table 6), suspected lung infection (Zhu *et al*[17] omitted, $I^2 = 0$, pooled RR = 0.78, 95%CI: 0.23-2.68, $P = 0.723$, Table 6), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.84, 95%CI: 0.33-2.18, $P = 0.969$, Table 6).

DISCUSSION

There were many clinical application of fingolimod due to its original medicine indication of reducing the migration of lymphocytes to the central nervous system, such as Alzheimer's disease and Susac syndrome[21,22]. Although the mechanisms of action of fingolimod in the treatment of AIS or ICH might be different, absorption of infarction lesion in AIS or hematoma in ICH both involved cytophagy *via* microglia in brain tissue or immunocyte in peripheral blood across injured brain-blood barrier[23,24]. Edema around lesion has probable results of microcirculation obstruction, injured brain-blood barrier, and inflammatory response caused by lymphocyte infiltration in stroke patients, which may strongly affect recovery of neurological function and prognosis due to its space occupying and toxic effect. Thus, fingolimod might reduce secondary damage after stroke *via* its pharmacological action. In our meta-analysis of RCTs, although we did not find that fingolimod could accelerate reduction of lesion

Table 6 Overall standard mean difference or pooled risk ratio *via* random-effects model and pooled risk ratio of remaining studies after the study with the highest quality is omitted

Method	Overall SMD or RR (95%CI)	Test of heterogeneity	
		I^2 (%)	*P*
Random-effects model			
$CD8^+$ T cells	-3.59 (-4.37, -2.80)	0.0	0.737
Lesion volume	-0.17 (-0.75, 0.42)	0.0	0.917
mBI score	2.43 (1.59, 3.26)	10.6	0.290
RR of fever	0.93 (0.37, 2.32)	0.0	0.864
RR of suspected lung infection	0.90 (0.33, 2.43)	0.0	0.876
RR of adverse events occurring at least once	0.82 (0.36, 1.87)	0.0	0.995
Article deleted			
Zhu *et al*[17] (RR of fever)	0.78 (0.23, 2.68)	0.0	0.723
Zhu *et al*[17] (RR of suspected lung infection)	0.78 (0.23, 2.68)	0.0	0.723
Tian *et al*[18] (RR of adverse events occurring at least once)	0.84 (0.33, 2.18)	0.0	0.969

SMD: Standard mean difference; RR: Risk ratio; $CD8^+$ T cell: T-lymphocytes with cluster of differentiation 8 expression; Lesion volume: Infarction or hematoma volume; mBI score: Modified Barthel index score.

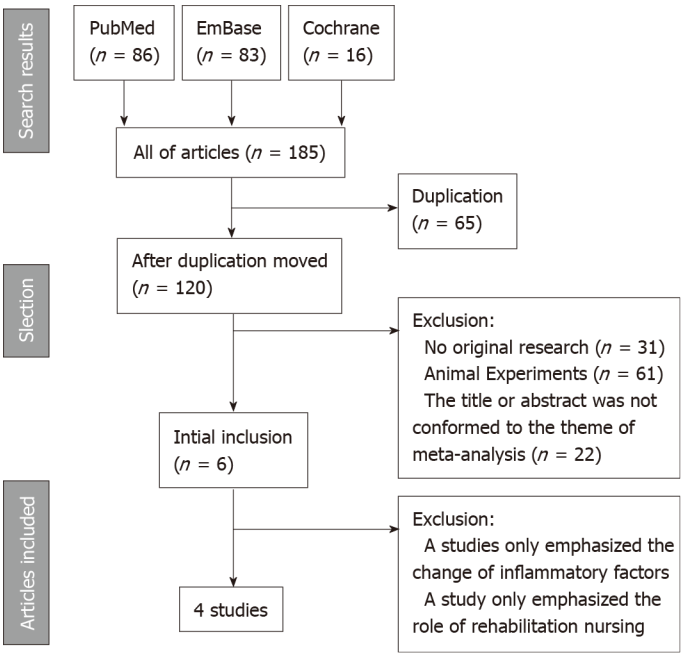


Figure 1 Process of study screening.

volume (I^2 = 0, overall SMD = -0.17, 95%CI: -0.75-0.42, *P* = 0.917, Figure 3) *via* reducing lymphocyte counts in peripheral blood (I^2 = 0, overall SMD = -3.59, 95%CI: -4.37--2.80, *P* = 0.737, Figure 3), there was an effective improvement in ADL in stroke patients (I^2 = 10.6%, overall SMD = 2.42, 95%CI: 1.63-3.21, *P* = 0.290, Figure 3), which might be the evidence that fingolimod might cause reduction of secondary damage *via* reducing lymphocyte infiltration in the brain effectively.

With regard to safety of oral fingolimod in stroke patients, infectious diseases might be the main adverse events. Especially, patients with hemiplegic paralysis may be aggravated to severe hypostatic pneumonia. In our meta-analysis, fever (I^2 = 0, pooled RR = 0.93, 95%CI: 0.97-2.32, *P* = 0.864, Figure 4) and suspected lung infection (I^2 = 0, pooled RR = 0.90, 95%CI: 0.33-2.43, *P* = 0.876, Figure 4) were both considered as infectious diseases. We did not find that fingolimod might increase the risk of

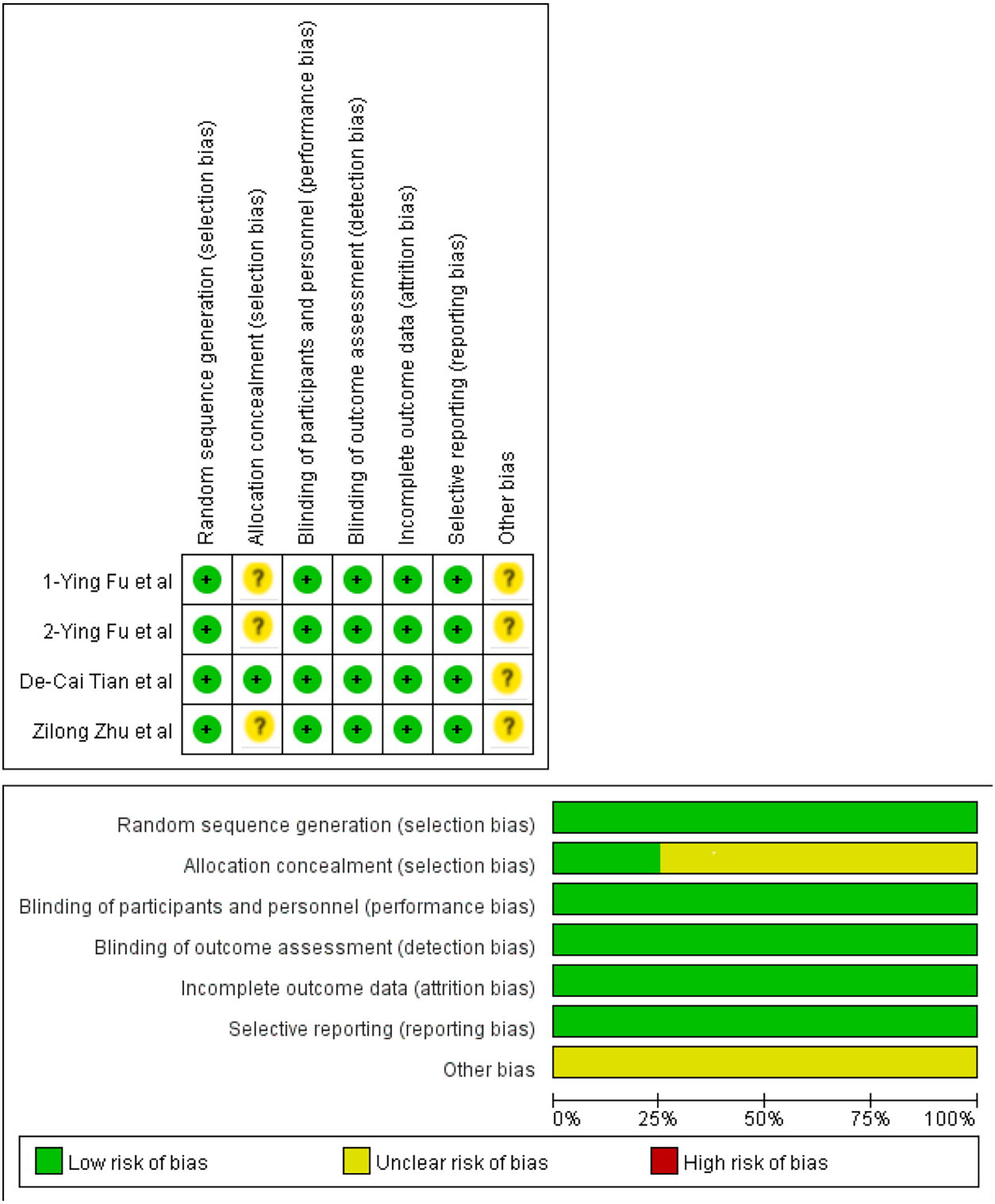


Figure 2 Quality assessment for studies included. 1-Ying Fu *et al*"[15] and 2-Ying Fu *et al*[16] represent two different references.

infection. Regarding other adverse events, there were some rare severe adverse events in the fingolimod group. Risk of cerebral hernia was not increased after the medication (RR = 0.35, 95%CI: 0.06-1.90, Table 3). The reason might be the large lesion volume at baseline. In the clinical trial by Tian *et al*[18], fingolimod was applied based on delayed alteplase administration. Hemorrhage of the digestive tract is definitely the potential risk during thrombolytic therapy. However, we found that the risk of digestive tract hemorrhage was not increased in the fingolimod group (RR = 0.42, 95%CI: 0.04-4.30, Table 3). Sphingosine-1-phosphate can regulate calcium ion movement in cells, which can affect myocardial contractility[25]. Therefore, fingolimod-phosphate can bind to sphingosine-1-phosphate receptors, which might result in arhythmia, such as bradycardia and atrial flutter. Likewise, sphingosine-1-phosphate can be produced

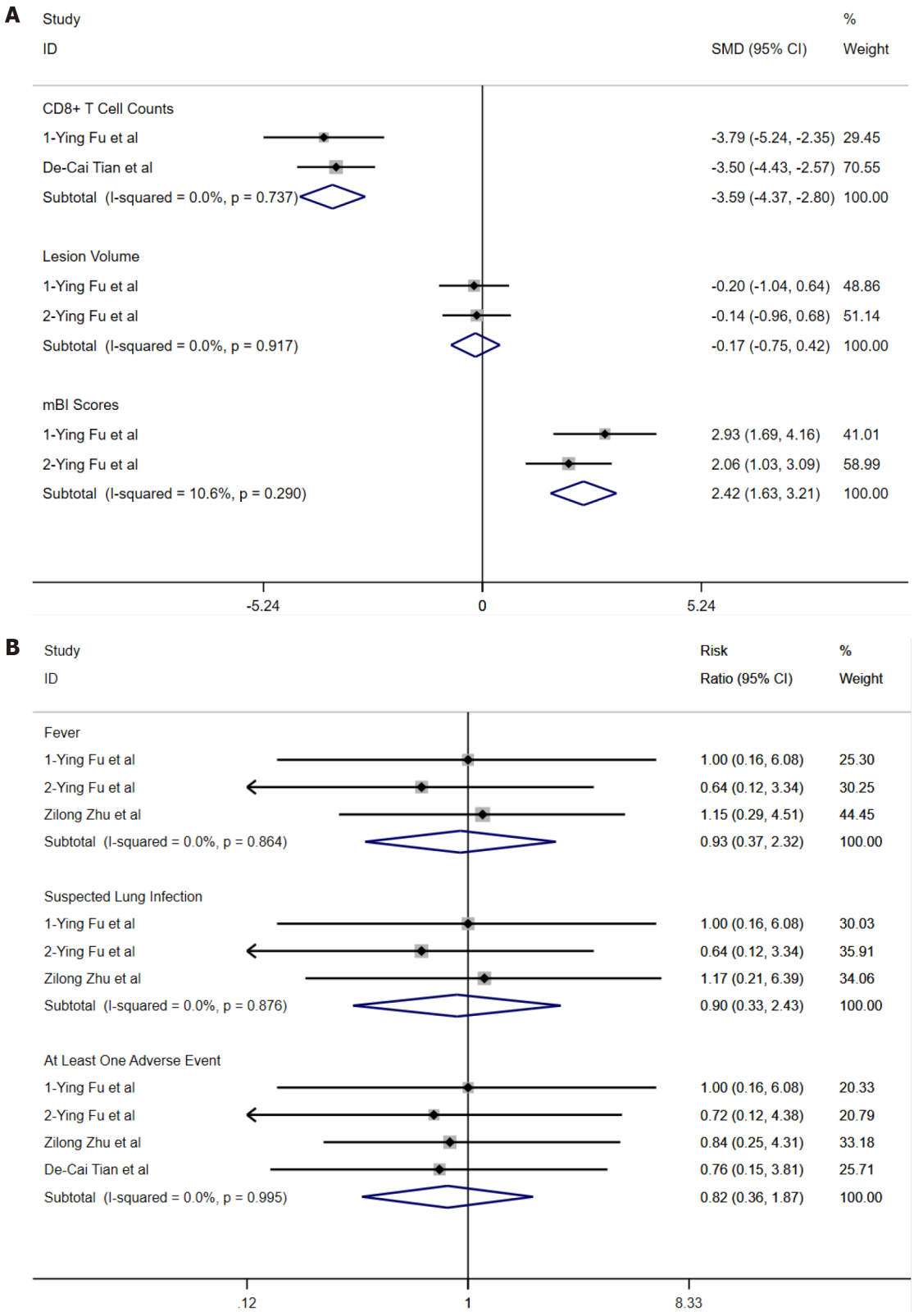


Figure 3 Forest plots of overall standard mean difference and pooled risk ratio. A: Overall standard mean difference of T-lymphocytes with cluster of differentiation 8 expression/infarction or hematoma volume/modified Barthel index scores; B: Pooled risk ratio of fever/suspected lung infection/adverse events occurring at least once. 1-Ying Fu *et al*"[15] and 2-Ying Fu *et al*[16] represent two different references. mBI scores: Modified Barthel index scores. RR: Risk ratio; CD8+ T cell: T-lymphocytes with cluster of differentiation 8 expression; Lesion volume: Infarction or hematoma volume; SMD: Standard mean difference.

from platelets and participate in platelet activation, which might result in thrombocytopenia in the fingolimod group[26]. Therefore, the application of fingolimod should be cautious in patients with a history of cardiac disease and platelet disorder. In general, according to pooled RR of adverse events occurring at least once (I^2 = 0, pooled RR =

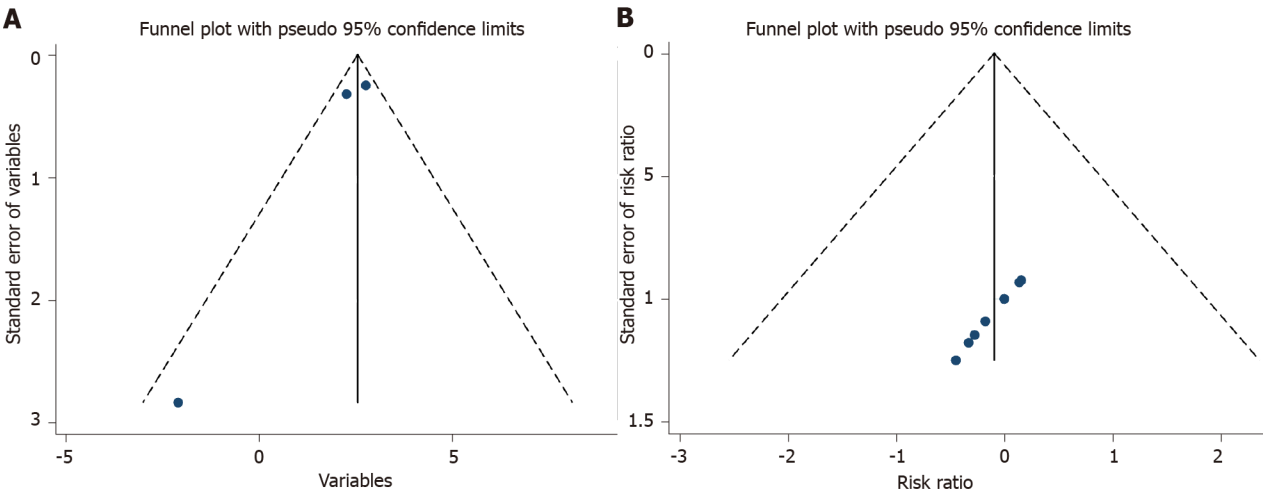


Figure 4 Funnel plots of publication bias. A: Funnel plot of variables used to acquire overall standard mean difference; B: Funnel diagram of risk ratio.

0.82, 95%CI: 0.36-1.87, P = 0.995, Figure 4), fingolimod might be safe for stroke patients.

Only four RCTs were included in the meta-analysis finally. Furthermore, two of them were completed by the team led by the same professor. The studies included was insufficient to perform a meta-analysis with high quality due to an insufficient number of patients. Although publication bias was not found, the outcome might be influenced by tendentiousness of articles with the same first authors. As some results were only shown in figures in some articles, these results were not extracted, which may lead to missing data at other time points and some variables, such as counts of other types of lymphocytes, edema around lesion, and NIHSS scores. In addition, there was potential influence of non-original data because data of continuous variables used in meta-analysis were processed *via* mathematical transformation owing to statistical difference before meta-analysis. Although a few stroke patients used fingolimod as a therapeutic medication, we believe that our research would bring about a new direction of stroke medication.

CONCLUSION

Fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively; however, we did not find the evidence that it could promote infarction lesion or hematoma absorption. In general, oral fingolimod (0.5 mg/d, 3 consecutive days) is safe in stroke patients except for some rare severe adverse events.

ARTICLE HIGHLIGHTS

Research background

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration.

Research motivation

The evidence-based of efficacy and safety evaluation of fingolimod in stroke patients is currently unavailable.

Research objectives

We hypothesized that fingolimod could effectively and safely promote reduction of infarction lesion or hematoma and improve neurological prognosis in AIS or ICH

patients by reducing lymphocyte infiltration.

Research methods

In this study, we performed a systemic review and meta-analysis of recent randomized controlled trials to confirm the above hypothesis.

Research results

There was a significant difference in $CD8^+$ T cell count and modified Barthel index between the fingolimod and control groups. However, there was no significant difference in lesion volume, fever, suspected lung infection (pooled RR = 0.90, 95%CI: 0.33-2.43, P = 0.876), and all adverse events occurring at least once between the fingolimod and control groups.

Research conclusions

Fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively. Fingolimod might not promote infarction lesion or hematoma absorption. Oral fingolimod (0.5 mg/d, 3 consecutive days) is safe in stroke patients except for some rare severe adverse events.

Research perspectives

More high-quality randomized controlled studies involving more patients are needed to provide more clinical research evidence.

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